THE ROLE OF SUBSTANCE P IN EARLY EXPERIMENTAL PARKINSON’S DISEASE

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CHAPTER 1:
INTRODUCTION
Parkinson’s disease (PD) was first described by James Parkinson in 1815 as “shaking palsy syndrome”. Today it is the second most common neurodegenerative disorder that affects 1-2% of the population over the age of 65, equating to more than 30 000 Australians. Unfortunately, the impact on public health can only escalate as our population ages, especially as the cause of PD remains unknown. PD is characterised by a loss of dopamine neurons from the basal ganglia, an integral part of the brain that creates smooth execution of movement. The loss of neurons occurs slowly over many years suggesting that a therapy could halt or slow down the progression of the disease. To date, no known neuroprotective therapy exists. In addition to the loss of neurons, there is a reduction in the expression of the neuropeptide, substance P (SP), an important neurotransmitter in the basal ganglia, which is essential for proper execution of basal ganglia function. However, this loss of SP was seen in animal and human studies that represented end-stage PD, and no studies examining early-stage PD have been published.

The current thesis demonstrates that in an experimental model of early PD, SP production is actually increased. Administration of an SP NK₁ receptor antagonist reduced dopaminergic cell death and associated functional deficits whereas increasing SP concentration exacerbated the disease progression. The early-stage increase in SP contributed to dopaminergic cell death through its effects on DA production and inflammatory processes, implying that the late-stage loss of SP may actually be secondary to dopaminergic neurone death. This thesis is therefore the first study to attribute SP/NK₁ initiated neurogenic inflammation in dopaminergic cell death in PD. Before discussing SP and its role in the pathogenesis of PD, this introduction will first describe PD and the processes implicated in dopaminergic degeneration.
1.1 Epidemiology of Parkinson’s Disease

PD affects 1-2% of the population over 65 years of age, with a lifetime risk of 1 in 45 of developing the debilitating disease (Alves et al., 2008; Schapira and Olanow, 2004). Unfortunately as our lifetime expectancy rises and the proportion of the elderly enlarges, the prevalence of PD can only increase (Przedborski et al., 2003). An example of this is Sydney, where over the last 40 years the prevalence of PD has gone from 66 to 780 per 100 000 (Chan et al., 2005). Apart from the loss of function in the affected population, there are substantial economic costs involved in the treatment of this disease. In Australia in 2006, it was estimated to cost around $7000 per PD patient per year who suffered moderate PD symptoms, which was four times greater than health care costs for a non-PD patient of the same age (Cordato et al., 2006). Thus with the rise in prevalence of PD over the next decades, PD and other neurodegenerative disorders are major public health problems with a large economic burden.

1.2 The Basal Ganglia

PD is characterised by a severe loss of the pigmented dopaminergic neurons of the substantia nigra (SN), which is an integral part of the basal ganglia (BG). The BG is interconnected masses of grey matter nuclei, located in the forebrain, midbrain and diencephalon. These nuclei are called the striatum, SN, globus pallidus (GP) and subthalamic nucleus (STN; Figure 1.1). The thalamus, although not a part of the BG, also plays a role in BG function (Yelnik, 2002). The BG primarily participates in the control of movement, consistent with the fact that the majority of its inputs are received from the motor areas of the cortex. These inputs are processed within the BG before a focused inhibitory output is sent back to motor areas of the cortex and to the brain stem (Squire et
al., 2003). However, the BG also includes the nucleus accumbens and ventral pallidum, which are part of the mesolimbic system and involved in motivational behaviour (Prensa and Parent, 2001). Thus lesions of the BG lead to movement disorders such as PD and Huntington’s disease (Hauber, 1998). The BG is an extremely complicated brain region that has many one-way and reciprocal connections between all the different nuclei. However for the current thesis, a simplified overview of the BG will be given.

1.2.1 Striatum

The striatum, which is named because of its stripy appearance, is located in the forebrain and receives the majority of BG input (Kramer and Mytilineou, 2004). The striatum receives both excitatory glutamatergic inputs from the cerebral cortex and thalamus, whilst at the same time receiving an inhibitory dopaminergic input from the SN (Parent, 1990). It also receives minor input from the GP and the STN (Graybiel, 1990). These inputs are topographically arranged within the striatum for focused functional outputs (Girault and Greengard, 2004). The striatum contains two chemically distinct regions, striosomes and the matrix. Striosomes are patches within the striatum, which are delineated by low levels of acetylcholine esterase (AChE) and high levels of substance P (SP) and dopamine (DA), with a core of intense tyrosine hydroxylase activity (Prensa et al., 2000). The striosomes receive information from the prefrontal cortex and send axon projections preferentially to the substantia nigra pars compacta (SNC). This pathway is known as the dopaminergic striatonigral pathway (Otsuka and Yoshioka, 1993). The bulk of the striatum is the matrix, which receives the majority of the input from the cerebral cortex and projects to the GP and substantia nigra pars reticulata (SNR), the major output nuclei of the BG (Snell, 1997). The matrix is composed of a large number of γ-
**Figure 1.1: Simplified flow diagram of the major basal ganglia pathways.**

Green arrows indicate GABA (inhibitory) projections, pink arrows indicate glutamate (excitatory) projections and blue arrows indicate modulatory dopamine projections. * denotes the major pathway between STN and GPe. Note; mesolimbic projections not shown. SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPi, Globus pallidus internal segment; GPe, globus pallidus external segment; STN, subthalamic nucleus. Modified from Squire et al., 2003.
aminobutyric acid (GABA)ergic medium spiny projection neurons, which contain either
SP, NKA and dynorphin (dyn) or enkephalin (Enk) and project to the SN and GP to
inhibit their function (Blanchet et al., 2000). These projection neurons constitute the
majority of neurons present within the matrix, as only 1-2% of the neuronal population
are interneurons (Bell et al., 1998). There are four types of interneuron’s: cholinergic,
GABAergic, which may contain either calretinin or parvalbumin, NOS interneurons,
which contain somatostatin, and NADPH-diaphorase interneurons (Pisani et al., 2003).
These interneurons are important in modulating BG function as they project to striatal
compartments to regulate the neuronal responses to DA input from the SN (Saka et al.,
2002).

1.2.2 Substantia Nigra

The SN is located in the midbrain and contains dopaminergic neurons. It is easily
recognised from surrounding tissue by its black pigmentation, called neuromelanin, a
residual product of normal DA metabolic activity (Burkitt et al., 1993). The SN is divided
into the SNC and the SNR. The SNC contains cell bodies of the dopaminergic neurons that
project to the striatum and thus are part of the striatonigral pathway. The SNC is of
particular importance for BG function, as striatal DA regulates the function of the other
basal ganglia nuclei (Prensa et al., 2000). Striatal DA is regulated by medium spiny
projections, which contain SP, neurokinin (NKA) and dynorphin. Furthermore, there is
direct regulation of the STN and GP by DA due to DA projections to the STN and GP,
which are present in both humans and rats (Prensa and Parent, 2001).

The SNR is one of the major output nuclei of the BG and is involved mainly in regulating
eye movement. It receives both inhibitory signals from the striatum and excitatory signals
from the STN, to execute smooth eye movements (Squire et al., 2003). The VTA is located adjacent to the SN and provides the DA input to the limbic system, for modulation of behaviour and emotion (Graybiel, 1990).

1.2.3 The Globus Pallidus

The globus pallidus (GP) is part of the lentiform nucleus and consists of an external (GPe) and an internal segment (GPi) as well as the limbic ventral pallidum. The GP receives inhibitory GABAergic signals from the striatum before itself using GABA as its main neurotransmitter to inhibit the activity of its target. It is the other major output nuclei of the BG and is responsible for limb movement (Squire et al., 2003) through both direct and indirect signalling pathways, which will be discussed further in 1.2.6. The GPi is involved in the direct signalling pathway whereas the GPe is involved in the indirect signalling pathway.

1.2.4 The Subthalamic Nucleus

The STN utilises glutamate as its neurotransmitter to excite its targets, the GPi and SN to increase their activity. Accordingly, the STN can modulate both burst firing and the firing rate of dopaminergic neurons in the SNC (Rodriguez et al., 1998).

1.2.5 Neurotransmitters in the Basal Ganglia

The BG contains a vast array of neurotransmitters and receptors including both classic excitatory and inhibitory neurotransmitters like glutamate, ACh, GABA and DA, and the neuropeptides SP, neurokinin A and B, all of which involved in the fine tuning of BG pathways (Graybiel, 1990; Hauber, 1998).
**Dopamine**

DA is an important neurotransmitter within the brain, especially in the BG. DA is unable to cross the BBB but its precursor L-DOPA, which is synthesised from tyrosine by tyrosine hydroxylase, readily enters the brain where L-DOPA decarboxylase almost instantaneously converts it to DA. Once released, DA may be taken back up into the presynaptic terminal and be metabolised by the enzymes monoamine oxidase-B (MOA-B) and alcohol dehydrogenase (ADH) to form 3,4-dihydroxyphenylacetic acid (DOPAC), which is further broken down by catechol-σ-methyltransferase (COMT) to form homovanillic acid (HVA). This is the main metabolic pathway of DA. Conversely, DA may be taken up by the postsynaptic neuron or into the extraneuronal uptake site, where it is metabolised by COMT to form 3-methoxytyramine (3-MT), then HVA by ADH or MAO-B (Figure 1.2) (Westerink, 1979).

DA receptors, D1 and D2, are metabotropic G-protein receptors, with D1 stimulating cyclic adenosine monophosphate (cAMP) whilst D2 decreases cAMP (van der Stelt and Di Marzo, 2003). Consequently, DA can activate cAMP dependent protein kinases, which can phosphorylate other receptors, ion channels and transcription factors or alter the permeability of K⁺ channels (Girault and Greengard, 2004). Within the BG, DA is fundamentally important in modulating BG function and therefore motor function due to its regulatory role on other neurotransmitter release and BG signalling pathways. Furthermore, in the limbic system DA is involved in reward processes and learning (Salamone et al., 2005). Thus abnormal DA transmission has been implicated in disease states such as PD, drug addiction, compulsive behaviour and schizophrenia (Aizman et al., 2000).
**Figure 1.2: Synthesis and metabolism of dopamine.**

The red pathway represents the main metabolic pathway and occurs when dopamine is taken back up into the presynaptic terminal, whereas the black pathway occurs when dopamine is taken up into the postsynaptic neuron or an extraneuronal uptake site. Diagram modified from (Clarke, 2004a).
**Glutamate**

Glutamate is the main excitatory neurotransmitter within the BG, with all known types of glutamate receptors such as AMPA, N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors, found throughout the BG including the SN (Vernon et al., 2005). In the striatum, both D1 and D2 receptor stimulation, together with SP and its receptor neurokinin 1 (NK1), stimulate striatal glutamate release (Marti et al., 2005). Dopaminergic neuronal activity is also partly modulated by glutamatergic inputs from the STN through AMPA receptors expressed on the soma of dopaminergic neurons (Chen et al., 2004). Also, on the terminals of DA neurons in the striatum there are both NMDA and non-NMDA ionotropic receptors, which when activated also excite the dopaminergic neurons to release DA (Morari et al., 1998). Conversely, DA can control the activity both AMPA and NMDA glutamate receptors by using PKA to phosphorylate these receptors and by regulating the activity of voltage-gated ion channels to inhibit the release of striatal glutamate (Girault and Greengard, 2004). Thus, glutamate and DA interact at both the pre- and post-synaptic levels to modulate cognition and motor functions (Morari et al., 1998). In PD, the metabolism of glutamate is increased in the striatum and SN, which may result in excitotoxic damage to DA neurons, as discussed in detail in 1.4.3. However, by restoring DA transmission back to basal levels, L-DOPA returns glutamate levels back to normal (Chassain et al., 2005).

**GABA**

GABA is the primary inhibitory neurotransmitter within the CNS and is found in approximately 30% of CNS neurons (Bowery et al., 2004). It is almost always co-localised with other neurotransmitters and is the most prominent transmitter within the BG (Graybiel, 1990; Di Cara et al., 2003). GABA is converted from glutamic acid by
glutamic acid decarboxylase and can be metabolised by GABA transaminase to succinic semi aldehyde, which can be further broken down to succinic acid (Wong et al., 2003). GABAergic neurotransmission causes long-term inhibition of synaptic transmission and can be either fast or slow depending on the GABA receptor subtype involved (Bowery et al., 2002). Fast transmission is mediated by GABA_A ionotropic receptors with ligand-gated chloride channels, and is the main receptor subtype found in the BG where it plays an integral role in control of movement. It has been shown to be important in anti-hyperkinesia (Varshavskaya et al., 2004). In contrast, slow GABAergic transmission is mediated by GABA_B receptors, which are metabotropic G-coupled receptors that reduce cAMP production and Ca^{2+} and K^{+} conductance to inhibit neuron activity. These are found in high numbers in the thalamus, cortex and dorsal horn of spinal cord (Bowery et al., 2002). In PD, excess GABA transmission may produce a lack of movement or difficulty in initiating movement. Furthermore abnormal movements produced by prolonged L-DOPA treatment may also be caused by excessive GABAergic transmission (Katz et al., 2005).

**Substance P**

SP is a major neuropeptide within the brain, and acts as an excitatory neurotransmitter within the BG (Napier et al., 1995). Particularly high levels of SP are found in the SN, the immunoreactivity of which accurately delineates this nucleus. SP is also present in the GP. Thus SP may be important in regulating the function of the SN and BG (Bell et al., 1998; Maubach et al., 2001). Like in other areas of the brain, SP is released in the BG due to a small elevation in Ca^{2+} (Otsuka and Yoshioka, 1993). Once released, it may bind to its NK_1 receptor on interneurons to depolarise membrane potentials then increase the firing rate causing the release other BG neurotransmitters such as GABA and ACh from
interneurons in the striatum (Aosaki and Kawaguchi, 1996; Kemel et al., 2002; Bailey et al., 2004). SP may also inhibit the release of ACh from cholinergic interneurons by stimulating DA release and subsequent activation of D2 receptors located on these interneurons, although for this to occur NMDA receptors have to be simultaneously activated (Blanchet et al., 2000). SP also plays a role in the limbic structures of the BG, for example within the nucleus accumbens, where SP via NK₁, increases DA release and reduces extracellular ACh (Kombian et al., 2003). NK₁ receptors are also located on 90% of DA neurons in the SNc (Chen et al., 2004), contrasting earlier reports that there was an absence of NK₁ receptors in the SN which created a mismatch between SP and NK₁ in this region (Humpel and Saria, 1993). This mismatch in the expression and binding of SP in the SN was subsequently shown to be due to the internalisation of the SP/NK₁ complex following SP binding, thus placing NK₁ receptors intracellularly (Levesque et al., 2007). SP can therefore directly cause the release of DA into the striatum (Orosz and Bennett, 1990; Reid et al., 1990a; Reid et al., 1990b; Galarraga et al., 1999). Furthermore, DA receptors are located on striatal projection neurons, supporting that DA or DA agonists can increase SP release (Humpel and Saria, 1993). Accordingly, an injection of SP into the BG area of rats induces behavioural effects such as sniffing, rearing, grooming and increased motor activity by potentiating striatal DA release (Saria, 1999). A reduction in striatal SP gene transcription and SP content in the SN in PD has also been observed, presumably due to the loss of striatal DA and therefore activation of SP-containing nigrostriatal projection neurons (Helke et al., 1990).

**Neurokinin A**

NKA is co-localised with SP in striatal projection neurons but unlike SP is mainly located in the matrix of the striatum (Kemel et al., 2002). It therefore has similar effects to SP in
the BG and has also been shown to also increase DA release (Reid et al., 1990b) by binding to NK₁ and neurokinin 3 (NK₃) receptors on dopaminergic neurons (Humpel and Saria, 1993; Levesque et al., 2007).

**Acetylcholine**

The striatum contains the highest concentrations of ACh, its muscarinic receptors, and other ACh markers within the brain (Hersch et al., 1994). The majority of ACh is located within cholinergic striatal interneurons, which receive both dopaminergic inputs from the SN and glutamatergic inputs from the cortex and thalamus, before signalling to both striatal projection neurons and interneurons (Pisani et al., 2003). For correct motor control, ACh and DA levels must be balanced, which occurs by D1 and D2 receptors, which inhibit ACh release (Blanchet et al., 2000; Calabresi et al., 2000). Therefore SP is also important in ACh regulation by promoting DA release. Consequently in PD, the loss of DA input leads to an elevation in ACh creating an imbalance of DA and ACh and improper motor control (Pisani et al., 2003).

**Enkephalin and Dynorphin**

The opioid peptides, enkephalin (enk) and dynorphin (dyn), and their receptors are expressed within medium spiny projection neurons that are involved in the striatopallidal and striatonigral pathways, respectively. Both peptides create a negative feedback mechanism to regulate the pathway in which they are contained (Steiner and Gerfen, 1999). Dyn modulates the response in these neurons by inhibiting DA release, however the exact mechanism by which enk works remains to be elucidated (Steiner and Gerfen, 1998). In PD, enk expression is increased within the striatum as a compensatory response to facilitate recovery from DA depletion and the subsequent lack of D2 receptor
activation (Steiner and Gerfen, 1999). Furthermore, elevated enk gene expression is suggested to be associated with dyskinesia following prolonged L-DOPA treatment (Henry et al., 2003).

1.2.6 The Direct and Indirect Signalling Pathways of the Basal Ganglia

The direct signalling pathway (Figure 1.3A) involves an excitatory glutamatergic signal being sent from the cortex to striatal GABAergic projection neurons that project to the GPi and SNr, resulting in inhibition of these nuclei and thus decreased inhibitory output to the thalamus. Consequently, the activity of thalamic neurons are increased causing excitatory glutamatergic signals to be sent back to the cortex, reinforcing cortical activity. The direct pathway therefore provides positive feedback for the cortex to allow movement (Silkis, 2001). Alternatively, the indirect pathway (Figure 1.3B) involves an excitatory signal being sent from the cortex to striatal inhibitory neurons that project to the GPe, resulting in decreased inhibitory output from the GPe to the STN. Subsequently, the STN sends excitatory signals to the GPi and SNr to increase their inhibitory output to the thalamus ensuring inhibition of the thalamic neurons and thus inhibition of cortical activity. The indirect pathway is therefore involved in negative feedback to the cortex and inhibition of the posture that was keeping the limb there (Sil'kis, 2002). These signalling pathways are kept in balance to ensure the almost simultaneous inhibition of the original position and initiation of the new required movement. Thus the direct and indirect pathways are involved in fine-tuning movement control and smooth execution of movement (Haber and Gdowski, 2004).
Figure 1.3: The direct and indirect signalling pathways within the basal ganglia.

The direct pathway (A) involves an excitatory signal being sent from the cortex to the striatal inhibitory neurons that project to the GPi and SNr, reducing their inhibitory output to the thalamus and therefore reinforcing cortical activity. The indirect pathway (B) involves an excitatory signal being sent from the cortex to the striatal inhibitory neurons that project to the GPe. Subsequently, GPe neurons produce less inhibitory output to the STN, thereby increasing STN excitatory output to the GPi and SNr, which in turn inhibit thalamic neuronal activity and cortical activity. SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPi, globus pallidus internal segment; GPe, globus pallidus external segment; STN, subthalamic nucleus; SP, substance P; NKA, neurokinin A; DYN, dynorphin; ENK, enkephalin. Pink arrows denote glutamatergic pathway. Green arrows denote GABAergic pathway. Diagram modified from (Haber and Gdowski, 2004).
A.

- Cortex → Thalamus (GABA)
- GABA → Striatum → GPi / SNr (SP, DYN, NKA) → Cortex

B.

- Cortex → Thalamus (GABA)
- GABA → Striatum → GPe → STN
- ENK → GPe → STN
- GABA → STN → GPe → Striatum → Cortex
The direct and indirect pathways are kept in balance by the dopaminergic input from the 
SNC to the striatal projection neurons, which express D1 and D2 receptors (Rouse et al., 
2000). Previous to initial thought, D1 and D2 receptors are co-localised in 99% of striatal 
projections neurons (Yelnik, 2002), although there are higher numbers of D1 than D2 
receptors on the projection neurons involved in the direct pathway and vice versa for the 
indirect pathway (Aizman et al., 2000). Therefore DA binds to D1 receptors, which 
increases activity by increasing cAMP production, to reinforce the activity of the direct 
pathway. In contrast, when DA binds to D2 receptors, cAMP production is reduced, 
creating a reversal of the activity of the nuclei within the indirect pathway. This results in 
increased activity of the cortical neurons and therefore reinforcement of cortical activity 
(Figure 1.4) (van der Stelt and Di Marzo, 2003).

Due to the loss of dopaminergic neurons in the SNC, and therefore a decrease in striatal 
DA, these two pathways become imbalanced in PD with an increase in the indirect 
signalling pathway and a decrease in the direct signalling pathway (Contreras-Vidal and 
Stelmach, 1996; Wardas et al., 2003). This imbalance creates abnormal levels of 
neurotransmitter release in PD within the signalling pathways. GABA, which is involved 
in both pathways, has increased release in the indirect pathway (Bianchi and Gray, 2002). 
Moreover, as enkephalin is the neurotransmitter involved with the indirect pathway and 
SP with the direct pathway, there is an increase in enkephalin and a decrease in SP 
expression in the striatum in both human and animal models of PD (Sivam, 1991; Nisbet 
et al., 1995; De Ceballos and Lopez-Lozano, 1999; Henry et al., 2003; Levy et al., 2005). 
The imbalance of the pathways produces the motor symptoms of rigidity, bradykinesia 
and akinesia (Rouse et al., 2000). Furthermore, the increase in the indirect pathway 
results in overactivity of the STN, which produces an increase in glutamatergic input to
**Figure 1.4:** Dopamine influence on the direct and indirect basal ganglia signalling pathways.

Dopamine is able to increase positive feedback to the cortex through the direct pathway and inhibit negative feedback through the indirect pathway. Black arrows indicate normal nuclei activity following increased cortical activity and the orange arrows indicate the activity of the nuclei under the influence of dopamine. SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPi, globus pallidus internal segment; GPe, globus pallidus external segment; STN, subthalamic nucleus; SP, substance P; NKA, neurokinin A; DYN, dynorphin; ENK, enkephalin; DA, dopamine. Diagram modified from (Haber and Gdowski, 2004).
the SN that will increase DA release or cause excitotoxic damage to further contribute to
the loss of DA neurons in PD (Rodriguez et al., 1998).

1.4 Mechanisms of Dopaminergic Cell Loss in PD

In PD, the severe loss of dopaminergic neurons is often accompanied by a loss of the
noradrenergic pigmented neurons of the locus coeruleus. This degeneration is a slow
progressive loss of about 5% per year (Blum et al., 2001). In the later stages of the disease
both the cholinergic neurons of the nucleus basalis of Meynert and the serotonergic
neurons of the dorsal raphe nucleus may also degenerate, although loss of these neurons
is not always seen (Marey-Semper et al., 1995). Along with the loss of neurons, formation
of Lewy bodies (LBs) are the pathological hallmarks of PD (Greenfields, 1992). LB
formation is also thought to contribute to dopaminergic cell death.

The cause of cell loss in PD is largely unknown, although mitochondrial dysfunction,
glutamate excitotoxicity, oxidative stress, and inflammation have implicated (Eriksen et
al., 2005). Furthermore, cell loss is thought to occur via apoptosis rather then necrosis
since this type of cell death is promoted by a variety of insults, some of which are seen in
PD (Olanow and Tatton, 1999).

1.4.1 Oxidative Stress

The vulnerability of dopaminergic neurons to insults may be due to the fact that under
normal circumstances they are already in a state of oxidative stress (Olanow et al., 2004;
Berretta et al., 2005). This is a result of high basal levels of reactive oxygen species
(ROS) due to enzymatic catabolism of DA by MAO-B, which produces H₂O₂ and the
normal non-enzymatic autooxidation of DA that results in neuromelanin (Schmidt and Ferger, 2001). Neuromelanin, seen as black pigmentation granules, is composed of DA-quinones and DA-semiquinones, which are lipid proteins that can bind iron (Fe\(^{2+}\)) and other metals (Fahn and Sulzer, 2004). Accordingly, in the SN of PD brains high amounts of Fe\(^{2+}\) are present (Zhang et al., 2005). Fe\(^{2+}\) in the presence of H\(_2\)O\(_2\) will convert to ferritin (Fe\(^{3+}\)) generating the highly reactive hydroxyl radical (OH\(^{•}\)) (Shults, 2004). Both H\(_2\)O\(_2\) and other ROS are powerful oxidising agents that cause oxidative damage to lipids, proteins and DNA (Elliot and Elliot, 1997). Indeed, by-products of oxidative damage are found in the SN of PD brains at post-mortem (Marsden and Olanow, 1998). As previously mentioned, there is an increase in DA turnover to compensate for the loss of DA neurons; consequently the production of H\(_2\)O\(_2\) and ROS are amplified causing greater oxidative stress. High ROS may lead to an increase in intracellular Ca\(^{2+}\) levels, which once elevated can be sequestered into mitochondria and initiate cell death cascades (Blum et al., 2001).

Unfortunately in PD, a loss of striatal DA terminals results in a loss of dopamine transporters (DAT), which creates excess extracellular DA that cannot be taken up into the cell and enzymatically degraded. Thus much of the DA undergoes autooxidation, which is more damaging to the cell as it produces DA-quinones and –semiquinones. Both of these are highly reactive and recently have been shown to induce microglia, the resident immune cell (Miyazaki and Asanuma, 2008; Zecca et al., 2008) that can be detrimental to the cell. Furthermore, nitric oxide (NO) is produced by NOS-containing interneurons and microglia contributing to the formation of reactive nitrogen species (RNS) such as peroxynitrite (ONOO\(^{−}\)). RNS species can be as damaging to dopaminergic neurons as ROS. Oxidative damage by these ROS and RNS is exacerbated in PD due to a deficiency of glutathione (GSH) and superoxide dismutase (SOD), the two main antioxidant enzymes that scavenge ROS, RNS and reduce H\(_2\)O\(_2\) to its non-reactive state.
(Canals et al., 2001). Also, in cell culture, a deficiency of GSH has been shown to exacerbate excitotoxicity (Beal, 1992).

1.4.2 Mitochondrial dysfunction

Mitochondria are involved in cell death as they produce high amounts of ROS during the uncoupling of electrons in the production of adenosine triphosphate (ATP). Additionally, they sequester and release Ca$^{2+}$ and Fe$^{2+}$, and integrate and amplify cell death signals (Stavrovskaya and Kristal, 2005). In PD there is a 30 to 40% decrease in complex I activity of the electron transport chain in mitochondria within the SN (Blum et al., 2001; Squire et al., 2003). This defect causes a reduction in the production of ATP, an important energy source for cell organelles, enzymes and transport systems. Thus, mitochondrial dysfunction causes a bioenergetic deficit that leads to membrane depolarisation, disruption of Ca$^{2+}$ homeostasis and production of free radicals (Shults, 2004). Specifically, a reduction in ATP causes impairment of the mitochondrial membrane potential and consequent opening of the mitochondrial permeability transition pore, which will result in the release of mitochondrial proteins such as cytochrome c and apoptosis-inducing factor (AIF) that signal apoptosis (Rego and Oliveira, 2003). Unfortunately aging also causes a decline in mitochondrial transport enzymes (Beal, 1992) therefore producing a cumulative effect on energy metabolism in PD. Furthermore, it has been demonstrated that mitochondria isolated from young rats were less likely to open the mitochondrial transition pore following pore opening events than middle or old aged rats (Stavrovskaya and Kristal, 2005).
1.4.3 Glutamate Excitotoxicity

With the loss of ATP production by mitochondria, there is a consequential loss of ATP-dependent magnesium-blockade of NMDA receptors, resulting in elevated levels of glutamate and NO (Chen et al., 1996). As previously mentioned, the SN dopaminergic neurons are rich in functional NMDA glutamate receptors and are therefore affected by any change in glutamate levels (Olanow and Tatton, 1999). Glutamate, an excitatory amino acid, causes an increase in intracellular Ca\(^{2+}\) resulting in free radical production, mitochondrial damage and activation of degradative enzymes like proteases, endonucleases and phospholipases. This results in degradation of plasma membranes, the cytoskeleton and nuclear material and subsequent cell death. This deleterious series of events, known as glutamate excitotoxicity, is thought to be one of the major contributors to cell loss in PD (Beal, 1992). It is further exacerbated by the reduced activity of calcium ATPase, which normally removes excess Ca\(^{2+}\) from cells (Schmidt and Ferger, 2001). In PD, glutamate excitotoxicity is thought to occur by an interaction between glutamate, DA and the D2 dopamine receptor in the striatum (Garside et al., 1996). Furthermore, a glutamate-mediated rise in Ca\(^{2+}\) leads to activation of NOS and thus an increase in NO. NO can produce the highly reactive OH\(^-\) by displacing Fe\(^{2+}\) from ferritin and by reacting with superoxide radicals (O\(_2^-\)) to produce ONOO\(^-\), another powerful oxidising agent (Guix et al., 2005). In addition, NO inhibits mitochondrial complex IV producing an irreversible respiratory chain defect (Olanow and Tatton, 1999). Once again, age may be a contributing factor to excitotoxicity in PD as the extent of neuronal damage and ability of neurons to recover from exposure to excitatory amino acids decreases with age (Wenk et al., 1995; Kannurpatti et al., 2004). Finally, due to the overactivity of the SN in PD, excess glutamatergic transmission to dopaminergic neurons can result in excitotoxic damage (Rodriguez et al., 1998)
1.4.4 Inflammatory Processes

Resting microglia are important for maintaining cellular homeostasis and once activated are involved in the removal of cellular debris (Mosley et al., 2006; Rock and Peterson, 2006). However, once microglia are activated, they also produce proinflammatory trophic factors, free radicals and cytokines such as interleukin-1 (IL-1), IL-2, IL-6 and tumour necrosis factorα (TNFα), which are potentially cytotoxic (Blum et al., 2001). Indeed, in human and in animal cases of PD there is a rise in these proinflammatory factors and cytokines in the SN, striatum and cerebrospinal fluid (CSF) (Jenner and Olanow, 1998; Liu and Hong, 2003). Normally, microglia produce ROS such as O$_2^-$ to kill foreign organisms that enter the brain. However, in the SN, where dopaminergic neurones already have reduced antioxidant capacity, production of free radicals could be catastrophic. Furthermore, microglia contain inducible NOS (iNOS), which once activated also secretes NO, which as previously established can result in the production of RNS. In the SN, large numbers of microglia are present compared to other areas of the brain, making dopaminergic neurons particularly sensitive to a variety of insults (Liu and Hong, 2003). Unfortunately, activation of microglia is a vicious cycle as the degeneration of neurons further triggers activation of microglia, thereby exacerbating the neurodegenerative cycle (Raivich et al., 1999).

In all animal models of PD, there is an increase in glial fibrillary acidic protein (GFAP) production in both the striatum and SN, with presence of activated, hypertrophic astrocytes (Depino et al., 2003; Takagi et al., 2007). These astrocytes can be both beneficial, due to their secretion of neurotrophic substances, glial derived nerve factor (GDNF) or brain derived nerve factor (BDNF), and detrimental by secreting proinflammatory cytokines (Hirsch, 2000; Brahmachari et al., 2006). These cytokines can
then activate additional microglia or astrocytes to further exacerbate the inflammatory response (Raivich et al., 1999; Chauhan et al., 2008).

Recently, blood brain barrier (BBB) dysfunction has been implicated in the pathophysiology of PD by allowing the influx of peripheral immune cells, such as blood borne macrophages, T-lymphocytes and leukocytes (Hunot et al., 1999; Kortekaas et al., 2005). Similar to central nervous system (CNS) immune cells, peripheral immune cells secrete cytokines following the translocation of transcription factor NF-κβ (Reale et al., 2008). An increase in cytokines, especially TNFα, can initiate apoptosis by binding to its receptor, tumour necrosis factor-α receptor1 (TNFαR1), a known death receptor, which is located on dopaminergic cell bodies in the SN (Mladenovic et al., 2004). Thus, any small insult in the SN could result in a full-scale immune response, ultimately causing cell loss by apoptosis. Therefore, peripheral immune cells not only directly injure dopaminergic neurons, they can also indirectly activate microglia and astrocytes to also contribute to the inflammatory injury and cell death.

**1.4.5 Apoptosis**

As mentioned earlier, cell death in PD is thought to occur via apoptosis. Apoptosis, also known as programmed cell death, occurs as a normal process that is involved in the development of the central nervous system (CNS), tissue homeostasis and elimination of unwanted or old cells (Cernak et al., 2002). It is characterised by cell shrinkage, formation of apoptotic bodies, condensation of chromatin, nuclear fragmentation and internucleosomal DNA fragmentation (Cotran et al., 1999). Nevertheless, cell death via apoptosis has been hard to prove due to the slow progression of PD, meaning that the number of cells undergoing apoptosis at anyone time is minimal (Olanow and Tatton,
However, markers of apoptosis such as caspase-3, caspase-1 and Bax, are increased in the SN, indicating that cell loss via apoptosis does occur in PD (Mochizuki et al., 1996). Furthermore, the presence of apoptotic cells and markers has also been demonstrated in animal models of PD (Burke and Kholodilov, 1998; Lev et al., 2003; Mladenovic et al., 2004; Przedborski et al., 2004).

1.4.6 Lewy Bodies

As mentioned earlier, filamentous cytoplasmic inclusions called Lewy bodies (LBs) are found in the surviving DA neurons of the SN. LBs are present in both idiopathic and familial forms of PD, except autosomal recessive juvenile onset PD (Meredith et al., 2004). Although LBs are found in other diseases such as diffuse Lewy body dementia and incidental Lewy body disease, they are considered to be the pathological hallmark of PD (Betarbet et al., 2005). LBs have a lipid core with filamentous periphery that gives a distinct halo appearance. The periphery contains a variety of proteins such as ubiquitin, cytoskeletal proteins, protein kinases, heat shock proteins, and proteins associated with inflammatory processes (Bennett, 2005). However, the main constituent of LBs is α-synuclein, a 140 amino acid neuronal presynaptic protein that is monomeric until it reaches high concentrations, at which point it aggregates into insoluble β-sheets (Meredith et al., 2004; Betarbet et al., 2005). Additionally, mutations of the α-synuclein gene, presence of Fe^{2+} and oxidative stress, all of which are present in PD, can cause α-synuclein to aggregate (Vila et al., 2000). It is this aggregation that is deleterious to the cell as it promotes secondary processes associated with cell injury including oxidative damage, excitotoxicity, inflammation and neurodegeneration via apoptosis and/or necrosis (Cummings, 2004). Interestingly, DA can bind to α-synuclein, stabilising it and
preventing it from aggregating, implying that a loss of DA in PD may exacerbate α-synuclein aggregation (Bennett, 2005).

1.4 Symptoms of Parkinson's Disease

PD has a slow progressive nature that takes many years of dopaminergic degeneration before symptoms such as resting tremor, bradykinesia and rigidity present themselves (Eriksen et al., 2005). Initially, dopaminergic cell loss in itself does not decrease striatal DA due to pre- and post-synaptic compensatory responses of the dopaminergic system. These include upregulation of D1 and D2 dopamine receptors, which have a lower threshold for activation than normal, and an increase in activity of the surviving dopaminergic neurons (Perez-Otano et al., 1998; Deumens et al., 2002). These compensatory mechanisms are able to sustain normal activity until approximately 50% of DA neurons and 80% of striatal DA are lost. It is at this stage that symptoms develop (Lee et al., 1996). Deficiency of DA in PD is heterogenous as DA is predominantly lost in the putamen area of the striatum. The putamen is involved in the motor function of the basal ganglia and therefore motor dysfunction is the predominant PD symptoms (Mokry, 1995; Kaasinen and Rinne, 2002). The most common presenting symptom is tremor (Guttman et al., 2003), which often begins in the extremities on one side of the body (Przedborski et al., 2003; Fahn and Sulzer, 2004). Bradykinesia, or slowness in movement, is often the most troubling symptom for patients, but fortunately responds well to treatment (Guttman et al., 2003). Other symptoms include muscle stiffness, postural instability, and swallowing and speech difficulties (Parkinson's Organisation of South Australia 2005). Furthermore, as mentioned previously, in the later stages of the PD, serotoninergic and cholinergic neuronal populations may degenerate. If these nuclei are affected, cognitive, autonomic and psychiatric problems may occur (Wolters, 2001;
Zgaljardic et al., 2004). In particular, dementia and depression are particularly common, affecting approximately 1/3 of PD sufferers (Deumens et al., 2002). Unfortunately, as there is no biological marker for the disease, diagnosis depends on neurological examinations, and since PD symptoms often begin in an insidious manner, early diagnosis is generally difficult (Sethi, 2002).

1.4.1 Measure of Outcome in PD

The Unified Parkinson’s Disease Rating Scale (UPDRS) is used to assess a PD patients’ quality of life, but can also be a good indicator of the efficacy of novel therapeutic agents in clinical trials. The UPDS composes of 4 parts; mentation, behaviour and mood; activities of daily living; motor function and control. It is quick to score and thus has been widely accepted for the evaluation of PD (Ramaker et al., 2002).

1.5 Parkinson’s Disease Risk Factors and Prevention

The aetiology of PD is largely unknown although head injury and exposure to environmental toxins such as carbon monoxide, manganese and pesticides have been linked to the disease (Liu et al., 2003). However, age is by far the greatest risk factor with the chance of developing PD escalating after 60 and continuing to rise as people get older. In contrast, both caffeine and nicotine from cigarette smoking are considered to reduce the risk of developing PD (Powers et al., 2008). Furthermore, chronic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of developing PD by up to 45% due to the extensive inflammatory reactions seen in PD (Chen et al., 2003).
Less than 10% of PD cases have a genetic disposition (Olanow and Tatton, 1999). Point or missense mutations of the \textit{\alpha-synuclein} and \textit{parkin} gene have been clearly linked to PD (Squire et al., 2003). These mutations generally induce PD earlier than sporadic cases, with age of onset being before 50 years. For example, point mutations and deletions in the \textit{parkin} gene may cause autosomal recessive juvenile PD (Orth and Tabrizi, 2003). However, the majority of cases are considered to be due to an interaction between genetic and environmental factors (Guttman et al., 2003; Schapira and Olanow, 2004).

\subsection*{1.6 L-DOPA Therapy in Parkinson’s Disease}

To date there is no cure for PD and no neuroprotective therapy that will halt or slow down its progression (Squire et al., 2003; Yuan et al., 2005). Instead, treatment involves managing patients’ symptoms. Normally this treatment is L-DOPA, the precursor to DA. The rationale for this therapy is to restore DA levels to near normal and therefore restore normal function of the basal ganglia for a period of time. Unfortunately, following prolonged use, many patients fail to maintain a good response and often experience “wearing off” effects or a reduction in time that L-DOPA alleviates symptoms, dyskinesia and psychotic symptoms (Krasnova et al., 2000). Motor complications like dyskinesia, or involuntary movements, occur in approximately 50-80\% of PD patients who have been on L-DOPA for more than 5-10 years, and are often more debilitating than the original motor deficits (Chen et al., 2004). There has been some debate about the whether L-DOPA actually contributes to neuron degeneration, since it has been shown to be toxic to cultured dopaminergic neurons by producing ROS through either oxidative metabolism or autooxidation of dopamine (Mena et al., 1992; Ziv et al., 1997). Nevertheless, there is currently no evidence in either experimental animals or clinical trials that L-DOPA is toxic and leads to neurodegeneration (Olanow et al., 2004).
Once PD sufferers have poor quality of life, with no or little symptomatic effect from L-DOPA, surgical interventions such as deep brain stimulation (DBS) or STN or GP lesions are their only option. It is these BG nuclei that become overactive in PD, therefore lesions inhibit their activity and reduce dyskinetic movement and akinesia (Hely et al., 2000). DBS provides a similar effect, however the exact mechanism of its action is unknown (Guttmann et al., 2003). These treatments do not, however, prevent the continued degeneration of dopaminergic neurons.

Due to success in experimental models, agents including MAO-B inhibitors, COMT inhibitor, DA agonists, co-enzyme Q10 (mitochondrial complex I) and antioxidants like vitamin E have been tried in clinical trials but unfortunately, apart from DA agonists, these treatments have had little or no success (Clarke, 2004b, a; Fahn and Sulzer, 2004). However, dopaminergic therapies produce side effects such as nausea, dizziness, insomnia and psychosis (Hely et al., 2000). Research into new protective or symptomatic treatment accordingly continues.

1.7 Experimental Models of Parkinson’s Disease

Animal models fill most of the required features of a PD model, even though their acute or subacute pathogenesis does not replicate the slow progression as observed in human PD. Moreover, the biochemical and molecular events of human PD are simply inaccessible without the use of experimental models. Not only do animals aid in the determination of PD pathophysiology but they may also provide insight into potential neuroprotective strategies (Orth and Tabrizi, 2003; Schober, 2004). The most common animal models of PD are the 6-OHDA rodent models and MPTP mouse and nonhuman
primate models of PD. Rodents are the most widely used species in research due to their small size, modest cost and the ease of using large numbers in one study (McIntosh et al., 1989). Although the rodent’s physiological responses are different from humans, and their brains are different in structure, the advantages are considered to outweigh the disadvantages (Polvishock et al., 1994).

1.7.1 Culture Models of PD

Cell culture models, particularly organotypic cell culture, are often used to determine biochemical and physiological pathways at a cellular level. Despite their obvious disadvantages, organotypic neuronal cultures form synaptic connections, establish neuronal-glial interactions, and retain the cytoarchitecture of adult tissue, thereby in part replicating an *in vivo* environment (Stoppini et al., 1991; Gahwiler et al., 1997; Schatz et al., 1999; Kress and Reynolds, 2003; Testa et al., 2005). Accordingly, organotypic culture is useful for studying the development of tissue morphology, physiology of tissue and for screening novel neuroprotective drugs (Gahwiler, 1981; Schatz et al., 1999). Organotypic culture of the ventral mesencephalon, which contains both the SN and the VTA, with striatal tissue has been particularly useful for mechanistic and dopaminergic cell survival studies.

1.7.2 6-OHDA Models

The 6-hydroxydopamine (6-OHDA) rat model of PD was developed in the 1960’s and remains one of the most widely used PD models today due to its reliability and reproducible nature (Blandini et al., 2008). Although it has a similar structure to DA, 6-OHDA is toxic since it is preferentially taken up by DAT into DA neurons, where it accumulates and then undergoes autooxidation, or is rapidly metabolised by MAO-B to
produce H$_2$O$_2$ and ROS (Schwarting and Huston, 1996). It also causes a reduction in the activity of GSH and SOD resulting in an inability of the brain to clear damaging free radicals (Schober, 2004). Thus, administration of 6-OHDA causes SN dopaminergic neurons to be under greater oxidative stress creating oxidative damage to lipids, proteins and DNA. 6-OHDA can either be injected to produce uni-lateral or bi-lateral lesions, although animals that are bi-laterally lesioned are often severely paralysed and generally unwell. Therefore it is the uni-lateral model that is the most commonly used 6-OHDA model. It has the great advantage of including quantifiable rotational behaviour that correlates with lesion size when animals are treated with DA agonists, apomorphine and amphetamine (Fornaguera et al., 1994; Yuan et al., 2005).

6-OHDA cannot penetrate the BBB and therefore must be injected stereotaxically into the animal’s brain allowing for the development of more than one type of 6-OHDA model. For a model that replicates end-stage PD, 6-OHDA is injected into the SNc directly or into the medial forebrain bundle (MFB). This model produces almost total loss of DA neurons, as well as some degeneration in the ventral tegmental area (VTA). Such a model would be useful for testing potential pharmacological and cell replacement therapies (Heikkila and Sonsalla, 1992). In contrast, when 6-OHDA is injected into the striatum, it causes an approximate 50% loss of DA neurons and 80% loss of striatal DA. This model characterises early and moderate PD and is useful for testing neuroprotective and neurotrophic therapies (Ichitani et al., 1994; Lee et al., 1996; Yuan et al., 2004, 2005).

*In vitro* studies using 6-OHDA have shown that 6-OHDA increases intracellular Ca$^{2+}$ within 30 minutes, as well as causing cell membrane hyperpolarisation and inhibition of
neuronal firing (Berretta et al., 2005). Similar to human PD, 6-OHDA treatment activates microglia cells stimulating production of IL-1β and TNFα (Depino et al., 2003; Mladenovic et al., 2004). Like all other PD models, 6-OHDA treatment also causes inhibition of mitochondrial complex I leading to mitochondrial dysfunction and cytochrome c release, and ultimately an activation of the intrinsic apoptotic cell pathway (Liang et al., 2004). It can also inhibit complex IV, resulting in an irreversible respiratory chain defect (Deumens et al., 2002). Moreover, 6-OHDA was shown to induce an early increase in p53, which promotes cell cycle arrest or apoptosis in proliferating cells and activation of Bax, a proapoptotic gene (Blum et al., 2001). However, despite endogenous 6-OHDA having been shown to accumulate in human PD (Schober, 2004), none of the 6-OHDA models result in the formation of Lewy bodies. Despite this, 6-OHDA remains a powerful research tool for the study of PD.

1.7.3 MPTP Models

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of “synthetic” heroin, was discovered in the late 1970’s when a young drug addict was hospitalised with a sudden onset of Parkinsonian symptoms, which responded well to treatment with L-DOPA (Kopin et al., 1986). This discovery led to the development of the systemic MPTP nonhuman primate model, which is considered to be the experimental model most similar to the human condition of PD, and a MPTP mouse model (Heikkila and Sonsalla, 1992). MPTP crosses the BBB and is converted to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by MAO. MPDP+ is then spontaneously oxidised to 1-methyl-4-phenylpyridinium (MPP+) in glial cells (Schmidt and Ferger, 2001). Once released MPP+ can either bind to vesicular monoamine transporters (VMAT) and be taken up into vesicles containing DA or bind to DAT on the plasma membrane and be taken up into the
dopaminergic neurons (Schober, 2004). Within DA neurons MPP⁺ accumulates in the mitochondria via energy-driven uptake and binds to complex I or II of the mitochondrial respiratory (electron) chain causing a reduction in ATP, an increase in glutamate, elevated intracellular Ca²⁺ levels and production of ROS (Przedborski et al., 2004).

MPTP shows species differences in toxicity, perhaps due to differing MAO-B activity, which creates varying amounts of MPP⁺ (Mokry, 1995). This may account for the lowered toxicity of MPTP in rats and greater behavioural deficits in MPTP treated primates compared with mice (Heikkila and Sonsalla, 1992). In a chronic MPTP mouse model where numerous low doses are administered over a period of weeks, there is an upregulation of α-synuclein within DA neurons, although aggregation into Lewy bodies was not observed (Vila et al., 2000; Petroske et al., 2001).

1.7.4 Rotenone Model

Rotenone, a widely used pesticide, is a highly specific complex I inhibitor (Sherer et al., 2003b). Like other complex I inhibitors it produces oxidative stress and damage to DNA, lipids and proteins due to high levels of ROS, which is exacerbated by a deficiency in GSH (Sherer et al., 2003c). Rotenone is extremely hydrophobic so is able to cross the BBB and other biological membranes to produce a systemic inhibition of complex I (Betarbet et al., 2000). Animals treated with rotenone had ubiquitin and α-synuclein inclusions in the SN, a modest depletion in ATP, activation of microglia and obvious motor deficits (Sherer et al., 2003a; Sherer et al., 2003c). Despite having many features of a good animal model of PD, animals have a variable response to rotenone treatment with only 50% of the animals showing degeneration of the SN (Orth and Tabrizi, 2003). In addition, due to the systemic nature of this model, many of the animals become very sick,
are unable to maintain normal feeding and grooming behaviours and are consequently unable to live for extended periods of time (Lapointe et al., 2004).

1.7.5 Transgenic and Knockout Mice Models of PD

Other animal models of PD include transgenic models in mice and drosophila, and knockout mice models including DA receptor, DAT, α-synuclein and parkin null mice. These models have been useful for characterising specific aspects of PD and are commonly used today, despite the recognition that transgenic and knockout animals frequently have genomic compensation that affects phenotypic expression.

Regardless of the experimental PD model used, they all share the a common theme of mitochondrial dysfunction causing a reduction in ATP production leading to elevated intracellular Ca^{2+} and release or activation of proapoptotic proteins causing degradation via apoptosis.

1.8 Substance P

Substance P (Figure 1.5) was first discovered in 1934 by Gaddum and Schild, as the active principle in a stable dry powder. In 1936, Von Euler suggested the peptidergic nature of SP as its activity was stopped following digestion with trypsin, although later it was discovered that the degradation of SP was due to chymotrypsin as SP is trypsin-resistant (Leeman and Ferguson, 2000). SP was the original member of the tachykinin family. Subsequently, NKA and neurokinin B (NKB) were discovered and also made members of this family. They share a common terminal sequence, Phe-X-Gly-Leu-Met-NH2, where X is Phe or Val (Saria, 1999; Harrison and Geppetti, 2001). This sequence is essential for their biological activity and thus there is a certain amount of cross reactivity.
among the tachykinin receptors and their ligands (Gerard et al., 1991; Khawaja and Rogers, 1996). SP is an undecapeptide that has greatest affinity for the NK1 receptor due to its proline (Pro) in position 8; Pro in position 8 adjacent to a crucial neutral or basic residue at position 7 will naturally prefer to bind to NK1 receptors (Severini et al., 2002).

\[
\text{Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH}_2 \\
\text{Substance P}
\]

\[
\text{Hys-Lys-Thr-Asp-Ser-Phe-Val=Gly-Leu-MetNH}_2 \\
\text{Neurokinin A}
\]

\[
\text{Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-MetNH}_2 \\
\text{Neurokinin B}
\]

**Figure 1.5:** Structure of the three mammalian tachykinins. Adapted from (Hokfelt et al., 2001).

1.8.1 Tachykinin Receptors

The NK1, NK2 and NK3 tachykinin receptors belong to the G-protein coupled receptor superfamily which consist of 7 hydrophobic transmembrane domains connected by extra- and intracellular loops and coupled to G-proteins (Harrison and Geppetti, 2001). NK1 and NK3 receptors are mainly found in the CNS, but are also present in peripheral tissues (Otsuka and Yoshioka, 1993). NK1 receptors are located widely throughout the brain with greatest immunoreactivity in the striatum, nucleus accumbens, hippocampus, hypothalamus and the raphe nuclei (Harrison and Geppetti, 2001). In contrast, NK3 receptors are most abundant in the cortex and on glia (Yip and Chahl, 2000). NK2
receptors are widely distributed in the peripheral nervous system (PNS) especially in smooth muscle of the respiratory, gastrointestinal and urinary tracts (Maggi, 1995).

SP binds to the hydrophobic ligand binding pocket within the extracellular loops of the NK₁ receptor causing rapid internalisation of the ligand and its receptor (Harrison and Geppetti, 2001). As NK₁ is a G-coupled receptor, the G-protein, which is coupled to adenylate cyclase, converts ATP to cAMP and in turn activates phospholipase C_β (PLC_β) (Saria, 1999). Activation of PLC_β will result in increased turnover of intracellular inositol 1,4,5-triphosphate (IP₃) and a subsequent elevation of intracellular Ca²⁺ (Gerard et al., 1991). The NK₁ receptor has a 5’ untranslated region containing a cAMP binding protein (CREB) that responds to elevated levels of cAMP and Ca²⁺ by increasing gene transcription of SP (Saria, 1999). This creates a positive feedback cycle for substance P. However, elevated levels of IP₃ and Ca²⁺ can also activate phospholipase A₂ (PLA₂) triggering the release of arachidonic acid (AA) from membrane phospholipids, in turn affecting inflammation and hemostasis. Conversely, SP can block potassium (K⁺) channels causing membrane depolarisation and/or activation of autoreceptors to inhibit its own release (Harrison and Geppetti, 2001). Furthermore, there is a truncated form of the NK₁ receptor, which is 311 amino acids instead of 407 amino acids long (Maggi, 1995), and like full length NK₁ is able to undergo desensitisation and internalisation once SP has bound, although it does not contain any C-terminal phosphorylated sites (Richardson et al., 2003).

1.8.2 Synthesis and Metabolism of SP

SP is synthesised from the preprotachykinin (PPT)-A gene, which also encodes NKA, neuropeptide K (NPK) and neuropeptide γ (NPγ), the latter two being elongated versions
Alternative splicing of the PPT-A gene results in 3 distinct mRNAs: α-PPT-A, β-PPT-A and γ-PPT-A, although all 3 PPT-A mRNAs encode for SP (Harrison and Geppetti, 2001). It has been shown that α-PPT-A mRNA is the main isoform of mRNA in the brain, whereas α- and γ-PPT-A mRNA are primarily expressed within the periphery (Severini et al., 2002). NKB is encoded by PPT-B gene and like PPT-A gene is conserved throughout species (Hoyle, 1998).

Synthesis of SP occurs at ribosomes in cell bodies of the dorsal root ganglia before it is packaged into large dense core vesicles with processing enzymes called convertases, which cleave at Lys-Arg, Arg-Arg or Arg-Lys bonds to release the active form of SP (Severini et al., 2002). When required, SP-containing vesicles undergo axonal transport to the terminal endings in both the CNS and PNS, where they undertake final enzymatic processing and post-translational enzymatic modifications such as C-terminal amidation (Hokfelt et al., 2000). As previously mentioned, SP release is triggered by a small rise in intracellular Ca$^{2+}$, which will increase the pH within the vesicle resulting in alkinisation and release of SP by exocytosis (Otsuka and Yoshioka, 1993). NK$_1$ receptors are also synthesised and then anterogradely transported along axons to peripheral and perhaps central terminals. Thus once SP is released it can activate the postsynaptic NK$_1$ (Malcangio and Bowery, 1999).

There are a number of enzymes that cleave SP but there are two main enzymes involved in the metabolism of SP, neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE) (Skidgel and Erdos, 2004). As NEP immunoreactivity in the CNS and PNS closely mirrors SP immunoreactivity, it is the major enzyme that metabolises SP and cleaves between Gln6-Phe7, Phe7-Phe8 and Gly9-Leu10 (Khawaja and Rogers, 1996).
ACE also cleaves SP at Phe8-Gly9, Gly9-Leu10 producing SP fragments SP (1-7) and SP (1-8), which are able to be cleaved again to form smaller peptides (Michael-Titus et al., 2002). ACE cleaves SP in the vasculature, urinogenital smooth muscle and CSF and is also localised in the striatonigral pathway, but degrades the fragments released from NEP (Strittmatter et al., 1984, Strittmatter, 1985 #66).

1.8.3 Distribution of SP

Tachykinins are located in capsaicin-sensitive neurons, also known as primary sensory neurons, within the CNS, peripheral tissue and non-neuronal cells including endothelial and inflammatory cells (Hokfelt et al., 2001). Within the brain there is an uneven distribution of SP with increased levels in grey matter. The highest concentrations of SP are found within the SN, the internal segment of the globus pallidus and the hypothalamus (Ribeiro-da-Silva and Hokfelt, 2000). Using a human brain mapping analyser, which has been reported to be more accurate than other quantitative methods, SP immunoreactivity in the SN was 25% higher in the pars compacta than the pars reticula with many SP immunoreactive fibers surrounded dopaminergic neurons in the SNc (Sutoo et al., 1999). SP has also been shown to be abundant in lamina I of the spinal cord (Benolieil et al., 2000) as well as in various regions believed to be involved in the control of learning, memory and emotional states, such as midbrain/striatum, hypothalamus, ventral pallidum, amygdala and hippocampus (Hasenohrl et al., 2000).

SP is co-localised with other neurotransmitters such as glutamate, GABA, DA, ACh and serotonin (5-HT), and neuropeptides, calcitonin gene-related peptide (CGRP) and galanin, and is able to regulate their release and/or inhibit their effects (Hasenohrl et al., 2000; Ribeiro-da-Silva and Hokfelt, 2000). SP is co-localised with glutamate, CGRP and
galanin in approximately 70% of terminals of primary afferent neurons in the dorsal horn, and thus are released in the response to the same stimuli (Malcangio and Bowery, 1999). CGRP increases the expression of NK₁ receptors and competes with SP for catabolism by endopeptidases and therefore enhances the bioavailability of SP (Seybold et al., 2003). NKA is co-localised with SP, but in smaller amounts, in intrinsic sensory neurons in the dorsal root ganglion and other areas of the brain such as the basal ganglia (Khawaja and Rogers, 1996). Within the dorsal root ganglia, excitability of the spinal cord by SP and NKA is greater than that of either peptide alone (Hokfelt et al., 2001).

1.8.4 Functions of SP

A rapid advance in the knowledge about SP and its functions was made possible by development of the non-peptide NK₁ antagonists, which are able cross the BBB and inhibit the effects of SP (Saria, 1999), and also through the development of NK₁ receptor knock-out mice (Hokfelt et al., 2000). These have enabled researchers to determine the role of SP and NK₁ receptors in disease states (Harrison and Geppetti, 2001). Not only does SP perform specific functions of its own, but due to its ability to regulate the release of other neurotransmitters such as glutamate, ACh and GABA, it is involved in a great variety of functions (Castro-Obregon et al., 2004).

SP is functionally available at all times, but is upregulated when required and during development (Hokfelt et al., 2001). It mediates such diverse processes as transmission of sensory information, smooth muscle contraction, nociception, sexual behaviour and possible wound healing and nerve regeneration (Gerard et al., 1991; Maggi, 1995). In addition, SP plays an important modulatory role in immunologic and inflammatory states, particularly neurogenic inflammation (Alves et al., 1999).
**Contractile Properties of SP**

As previously mentioned, when SP was first discovered it was its ability to contract smooth muscle that attracted attention. Smooth muscle is found in many areas of the body such as in the gastrointestinal, urinary and respiratory tracts and within blood vessels. Thus actions of SP include effects on gut motility, lowering blood pressure and increasing blood flow. It has also been shown to be important in respiration, with tachykinins being recognised as amongst the most potent bronchoconstrictors known (Otsuka and Yoshioka, 1993; Khawaja and Rogers, 1996). However this excitation and contraction of smooth muscle is mediated mainly through NK₂ receptors, although NK₁ does play a minor role (Harrison and Geppetti, 2001).

**Nociception**

SP also participates in sensory functions (Benolieil et al., 2000). NK₁ receptors are expressed at anatomical locations involved in processing noxious input to the spinal cord and once a nociceptive input has occurred, NK₁ undergoes regulation (Saria, 1999). Indeed upregulation of NK₁ receptors may modulate the intensity of chronic pain (Velazquez et al., 2002). Furthermore, NK₁ knock-out mice have altered nociception and can tolerate increased pain (Hokfelt et al., 2000).

**Neuronal Growth and Protection**

SP is involved in damage and growth related processes by inducing nerve growth factor (NGF) and fibroblast growth factor (FGF) to regenerate nerve endings *in vitro* (Sanberg et al., 1993; Nikolaus et al., 1997). Furthermore, the tachykinin receptor G protein is
involved in cAMP cascades and the inhibition of membrane Ca\(^{2+}\) channels, thus potentially attenuating Ca\(^{2+}\) influx or intracellular overloading and promoting neuroprotection, neuronal survival, neurite growth of neurons and reconstruction of synapses (Chen et al., 2004). The NK\(_1\) receptor may also have a possible role in synaptic plasticity associated with morphological and functional CNS development (Saria, 1999). SP may play a role in the response to neuronal tissue injury as it has been shown to enhance neuronal growth and survival of cells, as well as improve neuronal regeneration and functional recovery after neurotoxic lesions including amyloid β (Hasenohrl et al., 2000). Moreover, it has protected striatal neurons and D1 receptors from quinolol acid excitotoxicity both \textit{in vitro} and \textit{in vivo} (Calvo et al., 1996; Chen et al., 2004).

\textbf{Neurogenic Inflammation}

Neurogenic inflammation is a neurally elicited local painful inflammatory response characterised by vasodilation, protein extravasation and tissue swelling that can be induced by certain types of injury or infection (Vink et al., 2003). It is caused by a release of CGRP and SP from primary sensory nerve fibers surrounding blood vessels with subsequent activation of NK\(_1\) receptors on the endothelial cells of these vessels (Lever et al., 2003). CGRP, the most potent vasodilator, increases blood flow, bringing cytokines and inflammatory mediators to the area (Woie et al., 1993), whereas SP binding to NK\(_1\) receptors increases vessel permeability, leading to plasma extravasation and BBB breakdown (Hokfelt et al., 2001). In addition, SP facilitates the release of histamine from mast cells to further increase vessel permeability and extravasation (Alves et al., 1999). Thus, SP plays a central role in mediating extravascular migration of inflammatory cells into inflamed tissue (Harrison and Geppetti, 2001). Neurogenic inflammation has been well described in the periphery but just recently has been demonstrated within the brain.
following TBI and stroke (Nimmo et al., 2004; Turner and Vink, 2007), where it produced substantial blood barrier breakdown and subsequent oedema formation and functional deficits.

**Behavioural Effects of SP**

Due to the ability of SP to increase striatal DA content, it can induce motor activity such as locomotion and rearing. In the nucleus basalis of Meynert, SP activates NK$_1$ on cholinergic neurons and therefore is involved in the process of memory such as memory reinforcement and learning, (Nikolaus et al., 1999). In addition, SP and NK$_1$ have been implicated in neurological disorders such as schizophrenia, depression and anxiety (Chahl, 2006; Ebner and Singewald, 2006; Duzzioni et al., 2008). Accordingly, intracerebroventricular injections of SP increased anxiety in animals, with this effect blocked by administration of an NK$_1$ antagonist (Gavioli et al., 2002). Finally, SP has been associated with autonomic functions such as swallowing and sexual function (Hokfelt et al., 2001).

**Programmed Cell Death**

SP has been recognised as a potential contributor to apoptosis as it has been shown to activate caspase-3 in a cell free system (del Rio et al., 2001). However, in subsequent *in vitro* studies, SP and NK$_1$ have been demonstrated to induce non-apoptotic programmed cell death (PCD) that differs to apoptosis in morphology (Castro-Obregon et al., 2002). Non-apoptotic PCD is initiated when arrestin-2 interacts with the carboxyl-terminal of NK$_1$ following the binding of SP, inducing the MAP kinase activation pathway that involves Raf-1, MEK-2, ERK-2 to activate Nur77, a pro-death signal (Castro-Obregon et
This type of cell death has been implicated in Huntington’s Disease (HD), another neurodegenerative disorder, but it is unknown whether it is involved in cell loss in PD (Castro-Obregon et al., 2002).

**NK₁ antagonists**

NK₁ antagonists have been shown to have efficacy in treatment of depression, anxiety, stress and nausea after cytostatic drugs (Saria, 1999; Hokfelt et al., 2001). Recently, studies from our laboratory have shown that NK₁ antagonists have immense therapeutic benefits following TBI and stroke, as they reduce oedema formation and cell death, thereby significantly improving functional deficits (Turner et al., 2006; Donkin et al., 2007). Importantly, NK₁ antagonists have few side effects, as the antagonists are thought to only act on deranged systems with increased peptide release and not normal functioning systems (Hokfelt et al., 2000).

### 1.9 Previous Studies of Substance P in Parkinson’s Disease

#### 1.9.1 Human Studies

Understanding neurotransmitter changes in clinical PD is difficult because most of these types of studies are conducted post-mortem and therefore represent the very end-stage of the disease when progressional changes are complete and most of the DA neurons are lost. In addition, almost all of PD patients at this stage are on treatment such as L-DOPA, which restores levels of neurotransmitters back to normal (Nisbet et al., 1995; Levy et al., 2005). Nevertheless, post-mortem studies have shown that there is a loss of SP content in the striatum and SN (Mauborgne et al., 1983; Tenovuo et al., 1984; Sivam, 1991; Nisbet
et al., 1995; De Ceballos and Lopez-Lozano, 1999). Furthermore, there is a significant deficit of NK₁ receptors in the putamen and GP of PD patients compared to aged matched controls (Fernandez et al., 1994). In PD there is also a loss of SP-containing neurons in the pedunculopontine tegmental nucleus (PPTg). In a case of idiopathic PD, where the person died shortly after diagnosis from an unrelated cause, Lewy bodies were observed in surviving SP-containing neurons of the PPTg, suggesting that these SP neurons are affected early in PD (Gai et al., 1991).

1.9.2 Animal Studies

Like human PD, there are reports of a decrease in SP content in the SN and striatum in a variety of animal models (Bannon et al., 1995; Schwarting and Huston, 1996). However, in a study by Orosz and Bennet in 1990 using the 6-OHDA rat model of PD, it was shown that although there was a decline in SP-immunoreactivity and SP mRNA in tissue levels in the SN, there was a rise in SP-immunoreactivity in the extracellular space of the SN. Subsequently it was suggested that this rise was a compensatory mechanisms for the loss of intracellular SP (Orosz and Bennett, 1990), suggesting that because of the loss of DA neurons, active SP-containing terminals discharge SP into the extracellular neuropil of the SN, even in the face of reduced SP input overall in the model. Because of the loss of SP, the 6-OHDA animals were given replacement SP treatment into either the lateral ventricle or into the SN directly causing extra DA release into the striatum (Krasnova et al., 2000). Additionally, pre-treatment with SP promoted compensation of DA neurons and thus assisted in recovery of 6-OHDA treated rats (Nikolaus et al., 1997).

In the non-human primate MPTP model of PD, which arguably represents the model most similar to human PD, it was once again shown that there was a reduction in striatal SP
gene expression and therefore SP. This deficiency of SP correlated with the degree of motor symptoms present (Wade and Schneider, 2001). However if primates were treated with L-DOPA the decrease in SP gene expression was reversed (Herrero et al., 1995).

It has been shown that even peripheral administration of SP causes DA release in the striatum (Boix et al., 1992). For this reason, and the results with animal models of PD, it has been suggested to use SP or NK₁ agonists to treat human PD as a substitute for L-DOPA, which has side effects after prolonged use (Hasenohrl et al., 2000; Chen et al., 2004).

1.10 Synopsis

SP has been previously implicated in neurogenic inflammation and the induction of neuronal cell death following CNS insults. Despite the many studies of SP in PD, all of these studies have used either post-mortem clinical tissue or experimental animal models that have replicated end-stage PD where the dopaminergic degeneration has been quite advanced. No study has previously characterised the role of SP in early-stage PD where dopaminergic degeneration has only just begun. Accordingly, the current thesis will characterise the role of SP in early-stage PD using the rodent intrastriatal 6-OHDA model that replicates this phase of the disease. Our hypothesis is that SP will be increased during the early stages of PD and therefore will contribute to the deleterious processes that are involved in dopaminergic cell loss. Specifically, SP involvement in inflammatory processes and neurogenic inflammation will be investigated by administration of agents that would increase SP exposure, namely Captopril and SP, and also by inhibiting SP effects with administration of capsaicin and NK₁ antagonists. The subsequent functional deficits produced by 6-OHDA striatal lesions, and the potential effects of the
aforementioned treatments, will be assessed using a battery of functional tests that test overall motor, sensorimotor, akinesia, gait and neurological and behavioural outcome. Lesion size will be estimated from the ipsilateral turning response following amphetamine administration. Finally, quantification of dopaminergic cell loss and assessment of cellular integrity, nigral architecture, inflammatory responses and BBB dysfunction will be determined, and then correlated to functional outcome.

All methods will be detailed in chapter 2, with subsequent chapters focussing on experimental results including 6-OHDA treatment in vitro, and the effects of Captopril, capsaicin, SP and NK₁ antagonist administration following induction of in vivo 6-OHDA striatal lesions. Each chapter will be preceded by a brief introduction and summary of the experimental design, before providing detailed results. Since much of the discussion in the individual chapters and their interpretation relative to the overall hypothesis is relevant to each chapter and the final conclusions, there may be some overlap in the respective discussion of each chapter. Nonetheless, the overall conclusions of the work will be drawn together in the general discussion.
CHAPTER 2:
MATERIALS AND METHODS
2.1 Animals

2.1.1 Ethics

All experimental protocols presented in this thesis were conducted according to the guidelines established by the National Health and Medical Research Council and were approved by the animal ethics committee of the IMVS (64a/04 and 65/05).

2.1.2 Animal Preparation

Sprague-Dawley rat pups from postnatal days 5-7 were used for the \textit{in vitro} organotypic cell culture study. Pups were removed from their home cage and placed into a heated sheltered box until required. Adult male Sprague-Dawley rats weighing between 250-300g were used in the \textit{in vivo} studies. Animals were group housed in a conventional rodent room on a 12-hour day-night cycle with a standard diet of rodent pellets and water \textit{ad libitum}. All animals were rested for several days after delivery before inclusion in any experiment.

2.2 Experimental Procedures

2.2.1 Organotypic Slice Culture

All culture procedures were carried out under sterile conditions within a laminar flow hood. Similarly, all equipment was sterilized in an oven at 180° for 4 hours and all solutions were either sterile filtered, obtained pre-sterilized or autoclaved. All named chemicals were obtained from typical commercial sources unless otherwise stated.
Dissection of the Ventral Mesencephalon and Striatum

Organotypic slices were prepared from brains of P5-7 pups of either sex according to the procedure previously described by Gahwiler and Stoppini and colleagues, with minor modifications (Gahwiler, 1981, 1988; Stoppini et al., 1991). Briefly, pups were quickly decapitated and small dissecting scissors used to make an incision along the midline of the skull before the skull was prised open with forceps and the dorsal surface of the brain exposed. A spatula was then used to ease the brain from the skull onto a sterile chilled Petri dish, ventral surface upwards, before the Petri dish was placed under a dissecting microscope. To dissect out the striatum (ST), two incisions in the coronal plane were made; one through the forebrain anterior to the level of the optic chiasm and the second through the optic chiasm itself. This incised section of brain tissue was placed on its anterior surface and cortex and septum removed from the striatal tissue. Any meninges were removed with tweezers. The striatal tissue was placed on to a McIlwain tissue chopper where serial coronal sections of 300μm thickness were cut. Striatal sections were the gently teased apart in a Petri dish containing cooled Ringers balanced salt solution (154mM NaCl (Univar), 5.6mM KCl (Sigma P-4504), 2.25mM CaCl₂ (Sigma C-4901)).

A similar procedure was used to dissect the ventral mesencephalon (VM). A coronal incision was made through the posterior thalamus at the level of the mamillary bodies with a second incision made at the level of the pons to isolate the midbrain. The dorsal midbrain and cortical tissue were then removed from the ventral midbrain and the tissue placed onto the McIlwain tissue chopper where 200μm coronal sections were cut. The tissue was then transferred into a sterile Petri dish containing cooled ringers solution and the slices teased apart under a dissecting microscope.
For both tissues, up to 10 slices could be produced. The ST and VM slices were carefully transferred to separate sterile Petri dishes containing chilled Gey’s balanced salt solution (GBSS; Sigma G-9779) with 0.1% D-glucose (UniLab 267-598) and 0.1% potassium chloride (UniLab 504-200). The Petri dishes containing the tissue was placed at 4°C for 90 mins before mounting the slices.

**Mounting the Slices**

Slices of ST and VM tissue were placed onto round coverslips (Menzel-Glaser 13mm), which were autoclaved prior to use. A plasma clot of chick plasma and thrombin was used to mount the tissue slices onto the coverslips. A stock solution of chick plasma was prepared by adding 5mL of sterile purified water to lyophilised chicken plasma (Sigma, P-3266). This solution was then centrifuged at 2500 rpm for 30 mins and the supernatant sterile filtered before being frozen in 1ml aliquots. The thrombin solution was prepared by adding 0.1g of bovine thrombin (Sigma, T-4648) to 5mL of sterile purified water before centrifugation at 2500 rpm for 30 mins. The supernatant was sterile filtered and 200μL aliquots frozen.

On the day of culture, aliquots were removed from the freezer and allowed to defrost within the laminar flow hood. The thrombin aliquot was diluted with 5mL of sterile GBSS. Both chick plasma and thrombin remained on ice within the flow hood throughout the procedure. A 15μL drop of chick plasma was placed onto the coverslip before a slice of VM then ST was gently placed onto the coverslip in the chick plasma with a fine spatula. A 25μL drop of thrombin was placed onto the coverslip and carefully mixed with a sterile glass rod ensuring that once the plasma clot was formed the VM and ST tissue were no more than 1 mm apart. The coverslips were then carefully placed into a sterile
24-well plate (Falcon, 35-3047) and left for 30 mins before 0.5 mL of culture medium (50% DMEM, JRH Biosciences; 25% Hanks balanced salt solution with 10nM HEPES, IMVS Infectious Disease Laboratory Media Unit; 25% heat inactivated horse serum, JRH Biosciences; supplemented with glucose (1mL 50% D-glucose/100mL of medium), glutamine (500μL of 1mM L-gultamine/100mL of medium), and 0.5% gentamycin and penicillin (IMVS Infectious Disease Laboratory Media Unit) was added. Plates were placed into a 37°C incubator with 5% CO₂ (Sanyo CO₂ Incubator).

**Maintenance of Cultures**

After 4 days *in vitro*, cultures were treated with antimitotic agents cytosine-β-D-arabinofuranoside (Sigma, C-6645; 1ml/100ml of medium) and uridine (Sigma, U-3750; 1ml/100ml of medium) for 24hr to retard glial and fibroblast growth. The media was discarded at this time and fresh media added. Media was then changed every 3-4 days ensuring cultures remained sterile.

**Treatment of Cultures**

At day 19 *in vitro* cultures were treated with 0.6ml of medium containing either 6-OHDA (200μM (Sigma, H-116) with 200μm NMDA (Sigma, M-3262) and 10μM glycine (G-8898)), SP (10μM, Sigma, S-6883), NAT (10μM, Sigma A-6367) or a combination of 6-OHDA with SP or NAT for 1 hour. All treatment solutions were sterile filtered before being added to cultures. At 1hr post-treatment, media was removed and frozen at -80°C and fresh media added. Media was changed at approximately the same time each day for 4 days before fixation of cultures was performed. All removed media was frozen at -80°C then stored at -20°C.
Fixation of Cultures and Immunocytochemistry for Tyrosine Hydroxylase

Cultures were fixed by adding 1ml of 10% neutral buffered formalin solution (Fronine Laboratory Supplies, IMVS) to each well for 10 mins following removal of media. Cultures were then washed twice with phosphate buffered saline (PBS) before 1ml of PBS with 0.1% Triton X-100 (Sigma, T-9284) was added to the cultures for 15 mins to permeabilise the cells. Cultures were again carefully washed twice in PBS before 1ml of PBS with 0.5% hydrogen peroxide (H₂O₂, Merk, IMVS) was added to each culture for 15 mins to block endogenous peroxidases. Cultures were again washed twice in PBS before 0.5ml of 3% normal horse serum (NHS; Sigma, H-1270) was added for 15 mins to block unspecific binding. NHS was removed and tyrosine hydroxylase primary antibody (TH 1:4000, Chemicon, ab151) added to each culture and left to incubate at room temperature overnight. Following two washes with PBS, cultures were incubated with secondary anti-rabbit biotinylated antibody (Vector BA-1000, 1:250) for 30 mins at room temperature before being washed twice with PBS. Cultures were then incubated in tertiary streptavidin peroxidase conjugate (SPC; Pierce, 1:1000) for 1 hr at room temperature before being washed twice with PBS. Finally the immunocomplex was visualised with 3,3’-diaminobenzidine (DAB, Sigma D-8001) before cultures were counterstained with half strength haematoxylin and mounted with DPX. Cultures were visualised under light microscopy for TH immunoreactive neuronal growth.

2.2.2 6-OHDA Rodent Model of the Early Stages Parkinson’s Disease

Since this thesis was attempting to characterise the role of SP in early-stage PD, the intrastriatal 6-OHDA model of experimental PD was used. The 6-OHDA model of PD is widely used in experimental PD due to its highly reproducible nature (Blandini et al., 2008). After injection, 6-OHDA is readably taken up into dopaminergic terminals by
dopamine transporters resulting in a loss of dopaminergic terminals within the striatum. Subsequently, this loss of ST terminals results in delayed dopaminergic cell death within the SN, with a maximal loss of neurons seen at 1-2 weeks post-lesion (Lee et al., 1996). Approximately a 40% loss in dopaminergic cells in the right SN is observed, which produces minor neurological deficits that can be assessed with specific neurological tests.

**Anaesthesia**

**Halothane/Isoflurane**

Halothane (Rhone Merieux) and Isoflurane (Abbot Australasia) were obtained as volatile liquids from Lyppards Veterinary Supplies (Adelaide) and stored at room temperature in a drug safe protected from direct heat and light. General anaesthesia was induced by placing the animal into a Plexiglas induction chamber where 3% halothane/isoflurane was delivered in O$_2$ via a calibrated vaporiser at a flow rate of 1.5L/min. Anaesthesia was maintained throughout the surgery via a nose cone at 1.5-2% halothane/isoflurane delivered in O$_2$ at a flow rate of 1.5L/min.

**Lignocaine**

Lignocaine (Lignocaine hydrochloride, 2% Mavlab Australia) was supplied as an aqueous solution by Lyppards Veterinary Supplies (Adelaide) and stored at room temperature in a drug safe away from direct heat and light. Lignocaine was used as a local anaesthetic and a volume of 0.25 ml was administered directly onto the incision site of all animals following surgery.
**Induction of Experimental Parkinson’s Disease**

During the 12-hour day cycle, animals were removed from their home cages and anaesthetised as described above. Once a surgical level of anaesthetic was obtained, the animal was placed onto a thermostatically controlled heating pad in the prone position to allow maintenance of the animal’s body temperature during the short surgical procedure. Initially, the dorsal surface of animal’s head was shaved using small animal clippers and the incision area cleaned with an alcohol swab. When withdrawal to pain reflex was absent, a midline incision of approximately 1-2 cms in length was made on the dorsal surface of the head and the animal then placed into a rodent stereotaxic device (Benchmark AngleOne, Harvard Apparatus) using a 3.5cm foam block to ensure the animal was in a horizontal position. To secure the animal to the device, the animal’s upper incisor teeth were placed over the tooth bar set at -3.9mm before using ear bars to firmly secure the head in place. The skin and muscle were then retracted, the skull exposed and the area cleared so as to visualise the midline and bregma sutures. Two 0.7mm burr holes were made over the right striatum at the stereotaxic coordinates: (1) AP: 0.5mm, ML: 2.5mm, (2) AP:-0.5mm, ML:4.2mm relative to bregma (Lee et al., 1996) using a high-speed micro drill (Meisinger; Fine Science Tools). 2µL of 6-OHDA (5µg/µL; see section 2.3 for preparation) was then injected (0.5µL/min) into each of the above stereotaxic coordinates targeting the right striatum using a 5µL Hamilton micro syringe stereotaxically lowered 5.0 mm ventrally from the dura. The needle was then left in place for 2 mins before being slowly retracted. Following intrastralateral injections, the animal was removed from the stereotaxic device and anaesthetic delivery discontinued. A small amount of Betadine antiseptic solution and lignocaine was applied to the surgical area and the incision closed using 9mm surgical clips (Autoclip would clips, Becton Dickinson). An additional group of animals were subject to all surgical procedures except
for administration of 6-OHDA (sham surgery) and this group was used as a control group for all experiments.

**Post-Surgery Recovery**

Following surgery, all animals were placed on a thermostatically controlled heating pad and administered 3mL of 0.9% saline subcutaneously using a 23-G needle to prevent dehydration. Animals were monitored until they were awake and mobile at which time they were returned to their home cages.

**2.2.4 Perfusion Fixation**

Perfusion fixation was performed using 10% neutral buffered formalin (Fronine Laboratory Supplies, IMVS). At pre-selected time points following induction of PD, animals were anaesthetised (as described in 2.3.2) and placed in the supine position on a wire rack. When a surgical level of anaesthesia was achieved and pain reflexes absent, a bilateral thoracotomy was performed to expose the heart. A blunt 19-G needle was inserted into the left ventricle at the apex of the heart and advanced into the ascending aorta. Heparin (5000 I.U/1ml; David Bull Laboratories) was then slowly injected into the aorta, and the right atrium incised to allow vascular flushing of injected formalin. Perfusion was continued until the fluid from the right atrium ran clear after approximately 10 mins. Animals were left intact for at least 1h before the brain was removed and stored in 10% formalin for at least 1 week before processing.
2.3 Drug Preparation

Animals were randomly assigned to receive 6-OHDA plus either vehicle (saline) alone or with capsaicin, captopril, SP, n-acetyl L-tryptophan (NAT) or L-333,060. Specific details of the study design are provided in each chapter. For SP, NAT, L-333,060 or corresponding vehicle treatment, animals received an intracerebroventricular (icv) injection into the right lateral ventricle at stereotaxic co-ordinates -0.6mm AP; 1.5mm ML relative to bregma and -3.5mm ventrally from the dura (Paxinos and Watson, 1998) immediately after intrastriatal 6-OHDA injections. The second 0.7mm burr hole made at these co-ordinates facilitated injection. Captopril, capsaicin or corresponding vehicle injections were administered subcutaneously.

6-OHDA

6-hydroxydopamine-hydrobromide with 0.01% ascorbic acid (6-OHDA; Sigma, H-116) was stored at -20°C. When required, the compound was made up in 0.9% saline with the ascorbic acid being necessary to stabilise the 6-OHDA in solution. Due to the high reactivity of 6-OHDA to both light and oxygen, nitrogen gas was bubbled through the saline for 30 mins prior to use. The 6-OHDA solution was then used immediately, ensuring that it was kept on ice and away from direct light throughout the procedure. The 6-OHDA solution is no longer viable once it turns a red/amber colour, which is normally around 8 hours after preparation. A total dose of 20μg dissolved in 0.9% saline was administered at a concentration of 5μg/μL.

Captopril

Captopril (Sigma, C-4042) was stored at room temperature. A dose of 5mg/kg was dissolved in 1mL of 0.9% sterile saline and administered subcutaneously. This dose was
obtained from previous studies using captopril in experimental PD (Lopez-Real et al., 2005; Munoz et al., 2006). Specific details of the treatment regime are outlined in chapter 6.

**Artificial Cerebrospinal Fluid (CSF) Vehicle**

Artificial CSF was made up by dissolving 4.383g NaCl (150mM, UniVar, 465), 0.0999g CaCl₂ (1.8mM, Sigma, C-4901), 0.0722g MgSO₄ (1.2 mM, Sigma, M-7506), 0.1742g K₂HPO₄ (2.0mM, Sigma, P-5379), and 0.901g glucose (10mM, Sigma, G-728) in a total of 500 mL of distilled water and adjusting the pH to 7.4.

**Substance P**

Substance P acetate salt hydrate (SP; Sigma S-6883) was stored at -20°C. 0.5mg of SP was dissolved in artificial CSF at a concentration 5μg/μL. 2μL of SP solution was injected into the lateral ventricle using a 5μL Hamilton syringe at a rate of 0.5μL/min to give a total dose of 10μg of SP. The dose used was determined from Ukai and colleagues who reported physiological effects of SP at this dose (Ukai et al., 1995).

**N-acetyl-L-tryptophan (NK₁ antagonist)**

N-acetyl-L-tryptophan (NAT; Sigma, A-6367) was stored at 4°C. A 50μM stock solution of NAT was prepared by dissolving 0.00012g of NAT in 10mL of artificial CSF and adjusting the pH to 7.4 with NaOH. From the 50μM stock solution of NAT, 1μL was removed and added to 999μL of artificial CSF to give a dose of 50nM NAT. 2μL of this NAT solution was administered icv as for the SP solution. The dose used was determined from Bishop and colleagues who reported a physiological effect of NK₁ antagonists on behaviour at this dose (Bishop and Walker, 2004).
**L-333,060 (NK₁ antagonist)**

L-333,060 hydrochloride (TOCRIS Bioscience; 1145) was stored at room temperature. A 100μM stock solution of L-333,060 was prepared by dissolving 0.00043g of L-333,060 in 10 mls of sterile 0.9% saline before adjusting the pH to 7.4. 1μL of 100μM L-333,060 solution was added to 999μL of saline solution to give a 100nM dose of L-333,060. 2μL of this L-333,060 solution was administered icv as for SP solution.

**Capsaicin**

Capsaicin (Sigma, M-2028) was stored at 4°C. Animals receiving capsaicin were pretreated subcutaneously with capsaicin or equal volume vehicle at a dose of 125mg/kg over a 3 day period (50mg/kg day 1, 50mg/kg day 2 and 25mg/kg day 3) 14 days prior to induction of PD. Capsaicin was dissolved in 1ml of vehicle containing 20% alcohol, 20% Tween 20 (Sigma, P-7949), and 60% saline.

**2.4 Functional Assessment**

The basal ganglia is primarily involved in smooth execution of movement (Graybiel, 1990; Cenci and Lindgren, 2007), and as such 6-OHDA lesioned animals experience bradykinesia, sensorimotor deficits, akinesia, and gait abnormalities. Due to the mild nature of the experimental PD, a battery of functional tests is required to assess for functional deficits. In this thesis, the rotarod, bilateral asymmetry test, modified stepping tests and adjusting step test were used to assess motor function. Overall neurological deficit was also assessed using a modified neurological severity score while behaviour was assessed using the open field test. Due to the unilateral lesion in this model, animals exhibit circling behaviour following administration of a dopamine-releasing agent such as amphetamine, a stimulant of motor function. This circling behaviour was quantified using
the rotometer test and used to estimate the size of the lesion (Ungerstedt and Arbuthnott, 1970). All animals were assessed in random order twice a week commencing at days 3 post-lesion. Detailed explanations of the assessment procedure are outlined below.

2.4.1 Rotarod

The motor deficits associated with 6-OHDA unilateral lesions were assessed using the rotarod (Figure 2.1). The rotarod is one of the most sensitive tests for motor deficits in brain pathologies including PD, stroke and TBI (Hamm et al., 1994; Ogura et al., 2005). It is extensively used for mice and rats to test motor function including co-ordination and balance, and has been used to assess sensorimotor learning deficits (Ogura et al., 2005). Briefly, the rotarod consists of eighteen 1mm metal rods arranged in a circular assembly that rotates between 0-30 revolutions per minute (rpm). The sensitivity of the test is enhanced by the need for the animals to grip the narrow rods whilst walking. The animal is placed onto the stationary rotarod for 10 secs before the speed of the rotarod is increased by 3 rpm every 10-seconds until a maximum of 30 rpm was reached. The time that the animal remained walking on the rotarod was recorded (in seconds), with the task ending if the animal gripped on to the rotarod without walking for 2 complete revolutions, fell off the rotarod or completed the 2-minute trial. All animals were assessed at the same time each day (early morning) at days 3, 7, 10, 14, 17 and 21 post-lesion.
Figure 2.1: The rotarod.

The rotarod consists of a circular assembly of 18 x 1mm metal rods, which the animals walk on for up to 2 minutes. The speed of the rotarod was increased by 3 rpm each 10-second interval, with the speed controlled by the experimenter using a adjustable dial (bottom right corner)(rpm; revolutions per minute).
2.4.2 Bilateral Asymmetry Test

Also known as the sticky label test, the bilateral asymmetry test was used to measure sensory function following 6-OHDA lesions. It tests tactile extinction and sensory neglect (Schallert et al., 1982) and is suitable for unilateral 6-OHDA lesions as these animals experience sensory deficits on the contralateral side. Briefly, two strips of tape (1cm x 3.5cm; Sigma, L-8519) were applied to the saphenous (soft underside) of the forepaws (Figure 2.2). Time to removal for both the ipsilateral and contralateral forepaws was recorded. Each trial lasted a maximum of 120 seconds and animals were subjected to two consecutive trials, separated by a rest period. The average of the two trials was then used as their bilateral asymmetry test latency. All animals were assessed twice a week (days 3, 7, 10, 14, 17 and 21 post-lesion) for a 3-week period at the same time each day (early morning).

2.4.3 Stepping Tests

A modified version of the stepping tests described by Olsson and colleagues (Olsson et al., 1995) was used to determine stepping time and step length, or bradykinesia, and gait abnormalities. Prior to the start of the test, the animal’s ipsilateral hind paw was painted with a non-toxic water-based paint. The animal was then placed onto the ramp, which was suspended above ground, with the time taken for the animal to traverse the 60cm plank recorded as stepping time (Figure 2.3). The test was then repeated for the contralateral side. The paint on the animals’ hind paws allowed the number of steps and therefore step length to be determined. All animals were assessed at the same time each day (early morning) on days 3, 7, 10, 14, 17 and 21 post-lesion.
Figure 2.2: The bilateral asymmetry test.

The bilateral asymmetry test assessed sensorimotor function. Adhesive tape was placed on the animals’ forepaws as seen in this figure. Time taken to sense and remove the tape from each paw was recorded.
Figure 2.3: The stepping tests – stepping time and step length.

The stepping tests assessed gait abnormalities. Animals had to traverse along a suspended ramp, with stepping time and the number of steps recorded to allow determination of step length.
**Figure 2.4: The stepping tests - Initiation time and adjusting step test.**

Animals were held by the experimenter to ensure the hind part of the body and the forepaw not been tested were held above the surface, with the time taken to initiate movement in each forepaw recorded (A). In the adjusting step test, animals are held in the same position as in initiation time before their contralateral forepaw was moved slowly across the surface (B), with the number of adjusting steps was recorded. (Note: The black marks on the surface were the distance the animals were moved to assess contralateral forepaw adjusting steps).
Difficulty in initiation of movement or akinesia is a common symptom of PD, even in the early stages. For a simple test of initiation time, all animals were held such that the limbs not being tested were above the surface, with only the forelimb being tested permitted to make contact with surface (Figure 2.4.a). The time taken for the animal to move each of its front paws was recorded up to a maximum of 10 secs. However, the most sensitive test of akinesia in this model is the adjusting step test (Figure 2.4.b). This test required the animal to be held as before, with the ipsilateral forepaw touching the surface. The animal is then moved slowly sideways across the surface in a forehand then backhand direction (5 seconds for 0.45m). The number of adjusting steps was counted and recorded in both directions. The test was then repeated for the contralateral forepaw. All animals were assessed at the same time each day (early morning) at days 3, 7, 10, 14, 17 and 21 post-lesion. As the contralateral forelimb is affected in this test, these results are shown within this thesis.

2.4.5 Modified Neurological Severity Score

The modified neurological severity score (mNSS) has been successfully used to assess neurological outcome in middle cerebral artery occlusion models of stroke, which results in damage to striatal function (Melani et al., 1999). Accordingly, this mNSS was used in the current studies to assess neurological function following 6-OHDA unilateral lesions. The mNSS incorporates motor (abnormal movement, muscle status), sensory (proprioceptive, visual and tactile) and reflex tests (Li et al., 2000; Modo et al., 2000) where the higher the overall score the more severe the neurological deficits (Table 2.1). The rankings of the mNSS were as followed: 1-4 denotes mild injury, 5-9 moderate
injury, and 10-14 severe injury. Neurological function was assessed at the same time each day (early morning) on days 3, 7, 10, 14, 17 and 21 post-lesion.

<table>
<thead>
<tr>
<th>Behavioural Test</th>
<th>Score</th>
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<tbody>
<tr>
<td><strong>Motor tests</strong></td>
<td></td>
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<tr>
<td>Muscle status-Hemiplegia</td>
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<tr>
<td>Raising the rat by the tail:</td>
<td></td>
</tr>
<tr>
<td>Flexion of forelimb</td>
<td>1</td>
</tr>
<tr>
<td>Flexion of hindlimb</td>
<td>1</td>
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<tr>
<td>Head moving more than 10° to the vertical axis within 30 seconds</td>
<td>1</td>
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<tr>
<td>Placing the rat on the floor:</td>
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<tr>
<td>Inability to walk straight</td>
<td>1</td>
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<tr>
<td>Circling toward the paretic side</td>
<td>1</td>
</tr>
<tr>
<td>Falling down to the paretic side</td>
<td>1</td>
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<tr>
<td>Abnormal movements</td>
<td></td>
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<tr>
<td>Immobility and staring</td>
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<tr>
<td>Tremor (wet-dog-shakes)</td>
<td>1</td>
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<tr>
<td>Myodystony, irritability, seizures, myoclonus</td>
<td>1</td>
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<tr>
<td><strong>Sensory tests</strong></td>
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<tr>
<td>Visual and tactile placing (limb placing test</td>
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<td>to detect visual and superficial sensory)</td>
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<tr>
<td>Reaching the table slowly with limbs or can not place at all</td>
<td>1</td>
</tr>
<tr>
<td>Proprioceptive test (deep sensory):</td>
<td></td>
</tr>
<tr>
<td>Pushing the paw against the table edge to stimulate limb muscles</td>
<td>1</td>
</tr>
<tr>
<td>Reflex (blunt or sharp stimulation) absence of</td>
<td></td>
</tr>
<tr>
<td>Pinna reflex (a headshake when touching the auditory meatus)</td>
<td>1</td>
</tr>
<tr>
<td>Corneal reflex (an eye blink when lightly touching the cornea with cotton)</td>
<td>1</td>
</tr>
<tr>
<td>Startle reflex (a motor response to a brief loud noise from snapping a clipboard paper)</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2.1: The modified Neurological Severity Score.**

Animals were given a score of 1 in the mNSS if the movement was abnormal or reflexes were absent.
2.4.6 Open Field

The open field test was used to assess spontaneous exploratory behaviour following 6-OHDA lesions. The open field consists of a 1m x 1m enclosed area with 100 (10cm x 10cm) squares drawn onto the floor of the field (Figure 2.5). The animal is placed into the centre of field and allowed to explore for 5 minutes. The number of squares the animal enters is recorded, with the square being recorded twice or more if the animal left the square and then re-entered it. Also the number of rears, the time spent grooming (in seconds) and the time spent frozen (in seconds) was recorded. The animal was deemed to be rearing when both front paws were off the ground simultaneously, and was freezing when their whole body was completely motionless. Both of these behaviours, along with number of squares traversed, are indicators of the stress/anxiety level of the animal. However, the mild nature of 6-OHDA injury did not result in freezing and this activity was therefore not incorporated into the activity score. Activity score was thus determined by adding the number of squares traversed to the number of rears performed in the 5-minute period, after multiplying the latter by 10. There was a maximum of 250 points that could be awarded under this scheme and a ranking between 1-10 was produced where each 25-point increment was considered to be the next activity level; a score of 225 – 250 was awarded the maximal activity level of 10. Spontaneous exploratory behaviour was assessed at the same time each day (early morning) at days 3, 7, 10, 14, 17 and 21 post-lesion.
Figure 2.5: The open field.

The open field assessed spontaneous exploration following 6-OHDA striatal lesions. The open field consists of a 1 m by 1m enclosed area, with 100 10cm by 10 cm squares. The animal was left to explore for 5 mins with the number of squares traversed and the number of rears recorded, before being used to determine an activity level. To begin the test, animals are placed on the centre of the field (black dot).
2.4.7 Rotometer

The rotometer test has been widely used in experimental PD since the 1960s, particularly in relation to estimating lesion size in the unilateral 6-OHDA model (Ungerstedt and Arbuthnott, 1970). The rotometer (San Diego Instruments, CA) consists of frosted plastic spherical bowls and an automated counter, which counts both ipsilateral and contralateral rotations over a specified time period. The automated counter has a wire connector that is joined to the animal by an attached rubber band placed around the animal’s body just behind its front legs (Figure 2.6). Intrastriatal injections of 6-OHDA may result in circling behaviour depending on the size of the lesion. Circling ipsilaterally toward the lesion can be potentiated by administration of either a dopamine agonist or dopamine-releasing agent. As such, animals were administered the dopamine-releasing agent, D-amphetamine sulphate salt dissolved in 0.9% sterile saline (5mg/kg/mL; Sigma, A-5880) subcutaneously 10 mins prior to the beginning of the test. The animal was then secured on the rotometer to allow the number of ipsilateral rotations in 60 mins to be recorded, with a greater number of rotations signifying a larger lesion. All animals were tested on days 7 and 14 post-lesion.
Figure 2.6: The rotometer.

The rotometer estimated lesion size by recording the number of ipsilateral turns per minute during a 60-minute period following induction of motor activity by systemic amphetamine treatment. The animal was secured in harness placed behind its forepaws. This harness was then attached to an automated counter placed above the animal by a thin wire. A top half of the spherical container was present during the test, although it is not seen in the figure.
2.5 Histological Analysis

2.5.1 Perfusion Fixation and Brain Sampling

Following the specified survival period, animals were perfused fixed (as described in 2.2.4) and the brain removed and stored in formalin for at least 7 days before being processed for histology. Brains were placed in a rodent brain blocker (Kopf, PA002) and sectioned into consecutive 2mm coronal slices. Slices were then processed overnight in the Tissue-Tek VIP. Tissue processing was as follows: 20 mins each of graded ethanol baths (50%, 70%, 80%, 95%, 2x100%) followed by two 1.5hr xylene baths and finally paraffin baths of increasing time (30, 2x60, 90 mins). Brains slices were then individually embedded in paraffin wax and serial 5μm sections containing the striatum or substantia nigra were cut using a microtome (Microm). Sections were then stained as detailed in 2.5.2-2.5.7.

2.5.2 Haematoxylin & Eosin Staining

Haematoxylin & eosin (H&E) staining of brain sections was performed to allow observation of cellular changes within the lesioned brains. The staining was carried out as follows: slides were initially placed onto a hot blower to turn the wax molten before being placed into 2 changes of xylene (2 mins) for final de-waxing. Slides were then placed into 2 changes of ethanol (2 mins) before being placed into haematoxylin for 5 mins. Slides were washed well in running water and then differentiated in acid alcohol before briefly being washed again in running water and then placed into lithium carbonate for 5 secs. Slides were washed in running water and then placed in eosin for 1 min before being placed straight into 2 changes of ethanol (2 mins) then 2 changes of histolene (2
mins). Slides were cleared and mounted in DPX before being assessed using light microscopy (Olympus).

2.5.3 Immunohistochemistry for Tyrosine Hydroxylase

Sections were incubated with a polyclonal antibody specific for TH to enable visualisation of dopaminergic neurons, terminals and fibres within the SN and ST. Sections were de-waxed and dehydrated (as described in 2.6.2) before being placed in methanol with 30% H₂O₂ to block non-specific peroxidase activity. Sections were washed twice in PBS (2x3 mins) before citrate antigen retrieval was performed. Sections were allowed to cool to under 40°C before being washed in PBS (2x3mins) and then incubated for 45mins in 3% NHS (Sigma, H-1270) to block non-specific antigen binding. Sections were then incubated in TH primary antibody (Chemicon, ab151, 1:8000) at room temperature overnight. Once again sections were washed in PBS (2x3mins) before being incubated in an anti-rabbit biotinylated secondary antibody (Vector, BA-1000, 1:250) for 30 mins at room temperature and then washed in PBS (2x3mins). Sections were then incubated in the tertiary streptavidin peroxidase conjugate (SPC; Pierce, 1:1000) for 1 hour at room temperature and then washed again in PBS (2x3mins). Subsequently the immunocomplex was visualised using 3,3’-diaminobenzidine (DAB; Sigma, D-8001) as a chromogen in the peroxidase reaction. Sections were then assessed using light microscopy (Olympus). For TH immunoreactive cell counts within the SN, TH immunoreactive neurons were counted throughout the entire ipsilateral and contralateral SN, with the average of 2 counts taken. The positive TH cells in the ipsilateral SN were compared to the contralateral SN to negate any difference in the level of SN between the animals.
2.5.4 Immunohistochemistry for Substance P

Sections were incubated with a monoclonal antibody specific for SP to enable the visualisation of those neurons, glia and vessels expressing SP. Following the initial preparation of sections as for TH (2.5.3), EDTA antigen retrieval was performed and sections were incubated in 3% NHS for 45 mins before being incubated in SP primary antibody (Santa Cruz, sc-9758, 1:2000) at room temperature overnight. Sections were washed in PBS (2x3mins) before being incubated in anti-goat biotinylated secondary antibody (Vector, BA-9500, 1:250) for 30 mins at room temperature. Once again sections were washed in PBS (2x3mins) and then incubated in the tertiary antibody, SPC (1:1000) for 1 hr at room temperature before being washed again in PBS (2x3mins). The immunocomplex was visualised with DAB. Sections were then assessed using light microscopy (Olympus).

2.5.5 Immunocytochemistry for Glial Fibrillary Associated Protein

Sections were stained for GFAP to identify the astrocytic response in the ST and SN following 6-OHDA intrastriatal lesions. Following the initial preparation of sections as for TH (2.5.3), citrate antigen retrieval was performed and sections were incubated in 3% NHS for 45 mins before being incubated in a polyclonal GFAP primary antibody (Dako, Z0334, 1:40000) at room temperature overnight. Sections were washed in PBS (2x3mins) before being incubated in anti-rabbit biotinylated secondary antibody (1:250) for 30 mins at room temperature. Sections were once again washed in PBS (2x3mins) and then incubated in the tertiary antibody, SPC (1:1000) for 1 hr at room temperature before being washed again in PBS (2x3mins). The immunocomplex was visualised with DAB. Sections were then assessed using light microscopy (Olympus).
2.5.6 Immunocytochemistry for ED-1

Sections were stained for ED-1, which recognises the CD68 antigen, to detect the presence of activated microglia and macrophage in the ST and SN following intrastriatal 6-OHDA lesions. Following the initial preparation of sections as for TH (2.5.3), citrate antigen retrieval was performed and sections were incubated in 3% NHS for 45 mins before being incubated in ED-1 primary antibody (AbD Serotec, MCA341R, 1:400) at room temperature overnight. Sections were washed in PBS (2x3mins) before being incubated in anti-mouse biotinylated secondary antibody (Vector, BA-2000, 1:250) for 30 mins at room temperature. Once again sections were washed in PBS (2x3mins) and then incubated in the tertiary antibody, SPC (1:1000) for 1 hr at room temperature before being washed again in PBS (2x3mins). The immunocomplex was visualised with DAB. Sections were then assessed using light microscopy (Olympus).

2.5.7 Immunocytochemistry for Albumin

Sections were stained for albumin, to determine if blood brain barrier breakdown had occurred following 6-OHDA intrastriatal lesions. Following initial preparation of sections as for TH (2.5.3), sections were incubated with 3% NHS for 45 mins before being incubated with goat anti-rat albumin primary antibody (Cooper Biomedical, 0113-0341, 1:20000) overnight at room temperature. Sections were washed in PBS (2x3mins) before being incubated in anti-goat biotinylated secondary antibody (1:250) for 30 mins at room temperature. Following washing of sections in PBS (2x3mins), sections were incubated with tertiary antibody, SPC (1:1000) for 1 hour at room temperature before being washed with PBS (2x3mins). The immunocomplex was visualised with DAB and sections assessed using light microscopy (Olympus).
2.6 ELISA

2.6.1 Preparation of Brain Homogenate

At either day 3 or 7 post-intrastriatal 6-OHDA lesion, animals were re-anaesthetised with 4% isoflurane (1.5L/min O2) until a surgical level of anaesthesia was reached. The animal was then decapitated and the brain removed. The striatum and midbrain were dissected out as quickly as possible by placing the brain ventral surface upwards and making two incisions above and below the optic chiasm (Figure 2.7).

![Figure 2.7: Regions of striatum and SN used in ELISA.](image)

The SN was dissected out by making an incision along the red lines, then removing cortical tissue. The striatum was dissected out by making an incision along the blue line and removing the overlying cortex. A midline incision was made to separate the hemispheres.
Left and right brain regions were weighed and homogenised in 10 x brain region weight of homogenisation buffer (homogenisation buffer with 0.01% TritonX-100, DL-dithioreitol (DTT; Sigma D-9163) and protease inhibitors pepstatin A, aprotinin, leupeptin and phenylmethanesulfonyl fluoride (Sigma; P-4205, A-1153, L-9783, P-7626). Homogenised brain regions were kept on ice and vortexed every 5 mins for 20 mins before being centrifuged at 8500 rpm at 4°C for 15 mins. The supernatant was transferred to a clean eppendorf tube and frozen at -80°C.

2.6.2 Protein Estimation Assay

Supernatant from each brain region was rapidly thawed then added in triplicate (5μl/well) to a 96-well plate (Greiner Bio-One) along with triplicates of standards of known protein contents (0, 0.1, 0.2, 0.5, 1, 1.5, 2, 5, 7.5 and 10μg/μl of bovine serum albumin, BSA; Sigma T-8877). 20μL of reagent S (Biorad, 500-0115) was added per ml of reagent A (Biorad, 500-0113) then 25μl of this solution was added to each well. Finally, 200μl of reagent B (Biorad, 500-0114) was added to each well, and the reagents allowed to mix for approximately 20 mins. The plate was then read at 620nm on an Ascent Multiscan plate reader (Thermo Labsystems). Protein content of each brain homogenate sample was determined by producing a standard curve with the known protein content standards then using the equation of the trend line to determine the protein content within the unknown samples. Brain homogenate samples were then diluted with tris-buffered saline (TBS) to 400ng protein per 100μl of TBS.
2.6.3 ELISA for SP

An ELISA assay was used to determine the level of SP expression \textit{in vitro} following 6-OHDA treatment and in the ST and SN following 6-OHDA intrastriatal lesions. As such, samples of either medium from cell culture (20\(\mu\)L of media/80\(\mu\)L TBS) or protein isolated from whole brain (400ng protein/100\(\mu\)L TBS) was loaded into each well of a Maxisorp plate (Nunc) in triplicates. Blank wells with no loaded protein were included as controls. The protein was allowed to coat the wells overnight at 4°C. Samples were tipped off and blocking agent (0.2% gelatine solution for cell culture media and 0.5% gelatine solution for brain homogenate) added to each well until a meniscus formed before plates were given gentle agitation at room temperature for 1 hour. Wells were washed in TBS (3x) then incubated with 100\(\mu\)L of SP primary antibody (Chemicon, ab-1566, 1:1000) at 37°C for at least 1hr in a humid container. Following washes in TBS (3x), wells were incubated with 100\(\mu\)L of secondary anti-rabbit horseradish peroxidase conjugate (HRP; Rockford, 1:1000 for cell culture media, 1:500 brain homogenate) antibody for 1hr at 37°C oven in a humid container. Wells were washed in TBS (4x) and the liquid substrate system 3,3′-5,5′-tetramethylbenzidine (TMB; Sigma, T-8665) was used to reveal protein expression by adding 100\(\mu\)L to each well. The reaction was then stopped at the same time for each well with 50\(\mu\)L of 0.5M sulphuric acid (H\(_2\)SO\(_4\)). The level of SP expression was then determined by reading the absorbance at 450nm on an Ascent Multiscan plate reader.

2.6.4 ELISA for Lactate Dehydrogenase

An ELISA was used to determine the level of lactate dehydrogenase (LDH), which was taken to reflect cell death following 6-OHDA treatment \textit{in vitro}. Samples of medium from cell culture (20\(\mu\)L of media/80\(\mu\)L TBS) were loaded into each well of a Maxisorp plate
(Nunc) in triplicates. Wells with 400ng BSA/100μL TBS were included as a control. The protein was allowed to coat the wells overnight at 4°C. Samples were tipped off and blocking agent (3% BSA solution) added to each well until a meniscus formed. Plates were given gentle agitation at room temperature for 1 hour. Wells were washed in TBS (3x) then incubated with 100μL of LDH primary antibody (Abcam, ab-2101, 1:1000) at 37°C for at least 1hr in a humid container. Following washes in TBS (3x), wells were incubated with 100μL of secondary anti-goat HRP (Sigma, A-5420, 1:2000) antibody for 1hr at 37°C oven in a humid container. Wells were washed in TBS (4x) and the liquid substrate system TMB used to reveal protein expression by adding 100μL to each well. The reaction was then stopped at the same time for each well with 50μL of 0.5M H₂SO₄. The level of LDH expression was then determined by reading the absorbance at 450nm on an Ascent Multiscan plate reader.

2.7 Statistical Analysis

All parametric data was analysed using an Analysis of Variance (ANOVA) followed by Bonferroni post-tests. The non-parametric data was analysed using the Kruskal Wallis ANOVA followed by Dunn’s multiple comparisons test. All parametric data is expressed as mean ± standard error of the mean (SEM) and non-parametric data is expressed as the median.
CHAPTER 3:
THE EFFECTS OF SP AND THE NK₁ RECEPTOR ANTAGONIST,
N-ACETYL-L-TRYPTOPHAN, IN AN IN VITRO MODEL OF
PARKINSON’S DISEASE
3.1 Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease. It generally affects the elderly with 1-2% of the population over 65 having the disease (Alves et al., 2008). In PD there is known degeneration of dopaminergic neurons from the SN, with a subsequent loss of dopamine within the striatum. Both the SN and striatum are part of the BG, the region within the brain that is involved in smooth execution of motor functions. Although numerous neurotransmitters are expressed within the BG, modulation by dopamine is of upmost importance for it to correctly function.

The neuropeptide SP is found in the BG, in both the striatum and in particularly high levels within the SN, where it is involved in the release of DA. Therefore, SP like DA is important in modulating BG function. In PD a decrease in SP expression within the striatum and SN has been demonstrated (Mauborgne et al., 1983; Tenovuo et al., 1984; Nisbet et al., 1995; De Ceballos and Lopez-Lozano, 1999), with this loss of SP speculated to be involved in symptom presentation (Bannon et al., 1987; Barker, 1991; Wade and Schneider, 2001). However, these observations have been made in post-mortem tissue from known PD sufferers, and thus demonstrate the very end stage of disease progression.

Cell culture models are often used to determine biochemical and physiological pathways at a cellular level. Of particular use is the organotypic culture model, where tissue from the early postnatal period is cultured in an oxygen-rich environment either as a single tissue culture or combined with a target tissue region. This type of culture possesses numerous benefits such as its simplicity, capacity of cultures to survive long-term, ability of cultured neurons to form synaptic connections and establish neuronal-glial interactions,
but most importantly they retain the cytoarchitecture of adult tissue and therefore largely replicate *in vivo* environments (Stoppini et al., 1991; Gahwiler et al., 1997; Schatz et al., 1999; Kress and Reynolds, 2003; Testa et al., 2005). Accordingly, organotypic culture is useful for studying the development of tissue morphology, physiology of tissue and for screening novel neuroprotective drugs (Gahwiler, 1981; Schatz et al., 1999).

Organotypic culture of the ventral mesencephalon, which contains both the SN and the ventral tegmental area (VTA), with striatal tissue has been particularly useful for mechanistic and dopaminergic cell survival studies. Due to the controlled nature of the culture environment, determination of factors that result in dopaminergic cell death can be easily studied with use of various receptor agonists and antagonists. The aims of the present study were firstly to determine the effect of 6-OHDA treatment on initial SP expression *in vitro*, secondly, to investigate if addition of SP or the NK$_1$ receptor antagonist, NAT, with 6-OHDA treatment affects this expression and thirdly, to determine whether elevated levels of SP potentiates cell death *in vitro*.

### 3.2 Study Design

Sprague-Dawley rat pups from post-natal day 4-5 were used in the preparation of an organotypic cell culture of PD as described in Chapter 2.2.1. Previous studies have reported that this culture model more closely replicates *in vivo* models of PD than other *in vitro* models, which culture only one cell line (Kress and Reynolds, 2003). Initially, cultures were treated with the PD neurotoxin, 6-OHDA, for 0.5, 1 or 2 hours. The medium was then removed and SP content determined. In a second study, cultures were treated with 6-OHDA, SP, NAT or a combination of 6-OHDA with SP or NAT for 1 hour as described in detail in Chapter 2.2.1. Culture medium was then removed and fresh
medium added. The medium was then changed each subsequent day for 4 days. Using an
ELISA, SP content of the media was measured, as was LDH, a marker of cell death \textit{in}
\textit{vitro}. Immunohistochemistry for TH was performed to confirm the presence of
dopaminergic neurons.

3.2.1 ELISA

Culture media from all treatment regimes were assessed at 1hr, 1 and 2 days post-
treatment for SP content using an ELISA assay as described in Chapter 2.6.3. Similarly,
LDH content was determined in culture media at days 1, 2, 3 and 4 post-treatment using
an ELISA assay as described in Chapter 2.6.4.

3.2.2 Immunohistochemistry for TH

At day 4 post-treatment, media were removed and cultures stained for TH, a marker of
dopaminergic neurons, as described in Chapter 2.2.1.

3.2.3 Statistical Analysis

All data was assessed using analysis of variance followed by Bonferroni post-tests. All
data were displayed as mean ± SEM.
3.3 Results

3.3.1 Presence of Dopaminergic Neurons in Culture

All cultures had dopaminergic neurons present at day 4 post-lesion as determined using TH immunoreactivity. Some of the neurons showed some signs of degeneration, whereas other dopaminergic neurons had formed networks of synaptic connections (Figure 3.1).

Figure 3.1: Cultured dopaminergic neurons.
An example of TH immunoreactive (dopaminergic) neurons grown in nigro-striatal organotypic cultures. Note the synaptic connections between neurons.

3.3.2 SP Content Following Different 6-OHDA Treatment Times

All cultures exposed to 6-OHDA had significantly greater SP content than non-exposed control cultures ($p < 0.001$), indicating that 6-OHDA exposure in vitro generates an initial increase in SP production (Figure 3.2). No significant difference in content was observed between the 6-OHDA exposure groups ($p > 0.05$). For the subsequent experiments, we used a 1 h exposure time to 6-OHDA because this exposure resulted in maximal SP
content and minimal variability. This 1 h exposure time was consistent with other comparable \textit{in vitro} 6-OHDA studies reported by Kress and colleagues (2005).

![SP Content Graph](image)

**Figure 3.2: 6-OHDA treatment \textit{in vitro} - SP content following different 6-OHDA treatment times.**

6-OHDA treatment for 0.5h (light green), 1h (green) and 2h (dark green) produced a significant increase in SP content compared to the non-treated control cultures (orange). No significant differences in SP content were observed between different 6-OHDA treatment times (**\( p < 0.001 \)) compared to no toxin) (no toxin \( n=6 \), 6-OHDA \( n=6/\text{group} \)).

**3.3.3 SP Content Following 6-OHDA Exposure Combined with SP or NAT**

Control cultures not exposed to any compound had significantly higher SP content compared to blank control at all times post-treatment (\( p < 0.001 \)), supporting the concept that SP was being produced in organotypic culture. In the absence of 6-OHDA, treatment with the NK\(_1\) receptor antagonist, NAT, alone resulted in a slight increase in SP content at
1 hour post-treatment, although this increase was not statistically different from the control group (Figure 3.3). On days 1 and 2 post-treatment, this group had slightly reduced SP content. As expected, SP treatment alone resulted in a significant increase in SP content when compared to control at 1 hour post-treatment, since 10μM of SP was contained in the media that was used for assessment of SP content ($p < 0.001$). Nevertheless, despite the addition of fresh medium at day 1 post-treatment, this group still had elevated SP levels suggesting that there was some increased SP production by the tissue. This increase was not significant when compared to controls. By day 2 post-treatment, SP had returned to control levels. The 6-OHDA alone group had only a slight and non-significant elevation in SP content at both 1 hr and 1 day post-treatment compared to control, which was unexpected given that the preliminary study (Fig. 3.2) showed a significant increase in SP after only 0.5 hr of treatment. Similar to all other treatment groups, SP production had returned to control levels by day 2.

Combination treatment of 6-OHDA and NAT resulted in a comparable rise in SP content as the NAT alone group at 1 h after treatment. However, by day 1 post-treatment, SP production was significantly reduced in this group compared to the 6-OHDA alone group ($p < 0.05$), and even less than in the NAT alone group. At day 2, this combined 6-OHDA and NAT treatment group recorded the lowest SP content of all groups. In contrast, treatment with 6-OHDA and SP resulted in a markedly increased SP content at 1 hour post-treatment when compared to controls ($p < 0.001$). While there is the SP in the exposure medium to consider, the increase in SP was nonetheless greater than that observed for the 6-OHDA alone or the SP alone, suggesting that combined 6-OHDA with SP promotes further SP production. This effect was most apparent on day 1 post-
**Figure 3.3:** SP content following combined treatment of 6-OHDA with SP or the NK\textsubscript{1} receptor antagonist, NAT.

A non-treated control group (orange) was used as a baseline control for SP content. NAT treatment alone (red) had a minimal effect on SP content at 1 hr that returned to control levels by day 1. SP treatment alone (blue) produced a significant increase in SP content by 1 hr, and remained slightly elevated at day 1. Treatment with 6-OHDA alone (green) resulted in an increase in SP content at 1 hr and 1 day post-treatment, although this was not significant. Combination treatment of 6-OHDA with NAT (yellow) produced an initial rise in SP content at 1 hour, however by day 1 content, had declined to below control levels and was significantly less than 6-OHDA alone. In contrast when 6-OHDA was combined with SP (purple), content was significantly increased compared to control and 6-OHDA alone treatment groups at 1 hour, and remained significantly elevated at day 1. *** denotes $p < 0.001$ compared to control, ### denotes $p < 0.001$ compared to 6-OHDA alone; ** denotes $p < 0.01$ compared to SP alone (n=8/group/day).
A. **SP Content at 1h**

B. **SP Content at 1 d**

C. **SP Content at 2 d**
treatment, with the 6-OHDA + SP treatment group having significantly greater SP expression than control, SP and 6-OHDA alone treatment groups \( (p < 0.001) \). Similar to all other treatment groups, SP content in this group had returned to control levels by day 2 post-treatment.

### 3.3.4 LDH Content Following 6-OHDA Treatment Combined with SP or NAT

LDH content of control groups not exposed to any compound was significantly greater than the BSA control \( (p < 0.001, \) data not shown), indicating that organotypic cultures were producing LDH. NAT treatment alone did cause any significant increase in LDH compared to controls at any day post-treatment, although a slight elevation in LDH content was observed on days 2 and 3 (Figure 3.4). In contrast, the SP alone treated group had greater LDH content than controls, with a significant increase in LDH production observed on day 3 post-treatment \( (p < 0.05) \). However by day 4, LDH production had returned to control levels. 6-OHDA treatment alone resulted in a small increase in LDH content at day 1 and 2 post-treatment, with maximal LDH production on day 3 before returning to control levels by day 4.
**Figure 3.4:** 6-OHDA treatment *in vitro* – LDH content following combined treatment of 6-OHDA with SP or the NK₁ receptor antagonist, NAT.

Non-treated control (orange) was used as baseline control for LDH content. NAT treatment alone (red) produced a minimal increase in LDH content at days 2 and 3 post-treatment. SP treatment alone (blue) resulted in an increase in LDH content at days 1, 2 and 3 post-lesion, with a significant increase in LDH content at day 3 compared to control. 6-OHDA treatment alone (green) had peak increase in LDH content on day 3 post-treatment, although content was slightly higher than control on days 1 and 2. When 6-OHDA treatment was combined with NAT (yellow) no obvious increase in LDH content was observed compared to control throughout the recovery period. Conversely, combination of 6-OHDA and SP (purple) produced a highly significant elevation in LDH content at all days post-treatment, when compared to controls, SP and 6-OHDA treatment alone. *** denotes $p < 0.001$ compared to control, ### denotes $p < 0.001$ compared to 6-OHDA alone, ## denotes $p < 0.01$ compared to 6-OHDA alone, ••• denotes $p < 0.001$ compared to SP alone, • denotes $p < 0.05$ compared to SP alone (n=8/group/day).
Combined 6-OHDA with NAT treatment resulted in a small, non-significant elevation in LDH content on day 1 and 2 post-treatment, although production had decreased to control levels by day 3. However when SP treatment was combined with 6-OHDA, a highly significant increase in LDH production was observed at all times post-lesion compared to controls ($p < 0.001$). Furthermore, this group also showed significant increases in LDH content compared to 6-OHDA ($0.001 < p < 0.05$) and SP ($p < 0.001$) treatment alone at each day post-treatment. By day 3, LDH content in this combined group did not differ significantly from the SP and 6-OHDA+SP groups.

As observed with SP production, combination treatment of SP with 6-OHDA exacerbated cell death as measured by LDH production, more so than the addition of the individual treatments in isolation.

3.3.5 Comparison of SP and LDH expression

In treatments that resulted in increased SP content, LDH content was also increased suggesting that increased levels of SP are associated with increased cell death in vitro. (Fig. 3.5). Indeed, a significant linear correlation was present ($r=0.44; p = 0.002$; Fig. 3.6) between SP and LDH on days 1 and 2 in cultures exposed to either SP alone, 6-OHDA alone, or combined 6-OHDA plus SP. Furthermore LDH content remained elevated throughout the 4-day recovery period after exposure, particularly in the combined 6-OHDA and SP treated group, even though the organotypic cultures were no longer exposed to the compounds.
Figure 3.5: Comparison of SP and LDH content.

SP content at 1hr post-treatment (blue) tended to determine LDH content at 1d post-treatment (pink) as SP and LDH content displayed a similar trend in the different treatment groups, despite increased SP content.

Figure 3.6: Linear regression between SP and LDH content on days 1 and 2.

r=0.44; p = 0.002.
3.4 Discussion

6-OHDA, a hydroxylated analogue of dopamine, has been used to study PD since 1968 when Ungerstedt showed that an injection of 6-OHDA into the SN resulted in anterograde degeneration of dopaminergic neurons (Ungerstedt, 1968). Like DA, the compound is taken up into neurons by dopamine transporters (DAT) where it accumulates in the cytosol. It is then either rapidly deaminated by MAO or undergoes autooxidation, both of which result in the formation of H$_2$O$_2$ and ROS (Schwarting and Huston, 1996). Further increases in oxidative stress are caused by the ability of 6-OHDA to reduce striatal GSH and SOD activity, thereby impeding the scavenging of free radicals and further stressing the dopamine neurons (Schober, 2004).

6-OHDA can also partially inhibit complex I of the electron transport chain within mitochondria, leading to increased production of ROS and a reduction in ATP synthesis (Soto-Otero et al., 2000). Consequently, a reduction in Na$^+$/K$^+$-ATPase activity generally occurs resulting in neuronal depolarisation and inhibition of the voltage-dependent block by Mg$^{2+}$ on NMDA glutamate receptors. This permits an influx of Ca$^{2+}$ (Chen et al., 2004) whose high intracellular levels induce activation of proteases, endonucleases and phospholipases, resulting in damage to plasma membranes and eventual cell death. Previous *in vitro* studies have demonstrated that 6-OHDA treatment produces a rapid rise in intracellular free Ca$^{2+}$, which is sustained even after toxin washout (Berretta et al., 2005). Dopaminergic neurons are rich in NMDA receptors (Olanow and Tatton, 1999) and *in vivo* studies have demonstrated a protective effect of NMDA receptor antagonists, Group I metabotropic glutamate receptor (mGluR) antagonists, and Group II and III mGluR agonists, which inhibit neuronal glutamate release (Vernon et al., 2005). In deteriorating cultures exposed to 6-OHDA, glutamate levels are indeed elevated (Herrera-
Marschitz et al., 2000). The ability of 6-OHDA to inactivate glutamine synthetase, an ATP-dependent enzyme that converts potentially damaging glutamate to glutamine, may be one mechanism by which the compound elevates glutamate levels (Schor, 1988).

Elevated cytosolic Ca\(^{2+}\) can also activate Ca\(^{2+}\)-dependent neuronal nitric oxide synthase (nNOS) thereby generating NO. NO is an important biological signaling molecule and can be both beneficial and detrimental. Unfortunately in the setting of oxidative stress, it reacts with ROS such as the superoxide anion (O\(_2^-\)) to produce the powerful oxidant, peroxynitrite (ONOO\(^-\)) that damages DNA, RNA and proteins. Furthermore, NO has also been shown to increase DA release in vitro (Gomez-Urquijo et al., 1999) further contributing to oxidative stress. NO can also be produced by inducible NOS (iNOS), which is increased in both organotypic culture and experimental models of PD, as well as human PD (Hirsch et al., 1998; Katsuki et al., 2006; McGeer and McGeer, 2008). Indeed, in organotypic culture models of PD, high levels of NOS are found within striatal tissue, particularly near dopaminergic terminals (Shimizu et al., 2003). These results suggest that 6-OHDA mediated dopaminergic cell death can be mediated not only by oxidative stress, but also by glutamate excitotoxicity. Nonetheless, oxidative stress which produces lipid peroxidation, protein oxidation and DNA damage is thought to one of the major contributors to 6-OHDA-mediated cell death, and has been observed in both in vitro and in vivo models of PD (Sawada et al., 1996; Blum et al., 2001; Testa et al., 2005).

The neuropeptide SP is located in medium spiny projection neurons of the striatum and within the SN, where SP-containing projection neurons make synaptic connections with dopaminergic neurons. Thus SP, an excitatory neurotransmitter, causes release of DA by binding to its NK\(_1\) receptor located on the dopaminergic neurons. This produces
depolarisation and an increase in firing rate, facilitating the release of neurotransmitters such as DA and glutamate (Chen et al., 2004). DA can itself further increase the release of SP by binding to its receptors located on striatal medium spiny neurons to initiate release of SP from their synaptic terminals located near nigral dopaminergic neurons. Thus each neurotransmitter potentiates the release of the other. In the context of PD pathogenesis, SP may contribute to glutamate excitotoxicity by elevating glutamate and therefore intracellular Ca²⁺ levels, as well as further exacerbating oxidative stress by increasing DA and NO release from activated microglia.

In peripheral tissue, thrombin, a serine protease, cleaves protein-activated receptors (PARs) that are expressed on numerous cells, including neurons and astrocytes, to release SP and subsequently cause protein extravasation and oedema (de Garavilla et al., 2001). Although it remains unknown if thrombin does this in the CNS, SP has been shown to cause plasma extravasation and oedema following TBI and stroke (Turner et al., 2006; Donkin et al., 2007). In nigro-striatal organotypic culture, thrombin treatment caused microglia to produce NO leading to death of dopaminergic neurons and elevated LDH production (de Garavilla et al., 2001). As SP can activate microglia, it is possible that thrombin’s detrimental effects to dopaminergic neurons were mediated in part by SP.

As mentioned previously, organotypic culture models retain cytoarchitecture and make synaptic connections similar to those seen in vivo. Therefore as the nigro-striatal pathway is intact in these cultures, SP and DA interactions are comparable to those in vivo. As 6-OHDA is mechanistically similar to DA, the initial increase in SP by 6-OHDA observed in this study may be due to its ability to bind to DA receptors and thus initiate SP release. Unfortunately, in the SP agonist/antagonist study, 6-OHDA treatment did not produce the
expected initial increase in SP release. This may be due to the unstable nature of 6-OHDA, which may have been partially metabolised before the cultures were treated, or because the 6-OHDA-solute had been partially oxidised despite the routine preparation of the compound under anaerobic conditions. Nonetheless, 6-OHDA did cause some detrimental effects and regardless of the addition of SP to the media initially, the increase observed in the 6-OHDA+SP group was greater than the SP alone group. This suggests that 6-OHDA with SP has a synergistic effect on SP release. SP also remained significantly elevated in this group at day 1 following treatment compared to control and 6-OHDA treated groups, despite the removal of the toxic compounds. Activation of the SP NK₁ receptor, a G-coupled receptor, initiates a biochemical cascade that results in increased turnover of intracellular inositol 1,4,5-triphosphate (IP₃) and elevation in intracellular Ca²⁺. Located on the NK₁ receptor is a cAMP binding protein (CREB) that responds to elevated cAMP or Ca²⁺ by increasing gene transcription of SP, creating a positive feedback for SP (Saria, 1999). This may explain the continued elevation of SP in the SP, 6-OHDA and 6-OHDA+SP groups. Inhibition of this pathway by the NK₁ receptor antagonist, NAT, resulted in a decrease in SP expression in the NAT and 6-OHDA+NAT groups by day 1 post-treatment.

LDH production, a widely accepted marker of *in vitro* cell death, showed similar trends to that observed with SP expression after exposure to the various compounds. Again, the combined 6-OHDA and SP group demonstrated the greatest production of LDH, although exposure to SP alone also had significantly higher levels of LDH, particularly at days 2 and 3 post-treatment. Nonetheless, the combined 6-OHDA with SP showed significantly greater levels of LDH production than the SP alone group, suggesting that higher than basal levels of SP may cause cell death in nigro-striatal organotypic cultures.
Unfortunately, it cannot be definitively stated that elevated levels of SP is specifically associated with dopaminergic cell death as increased LDH is a non-specific marker of tissue injury and in these cultures dopamine neurons are only a small subset of the total population of cells. However, dopaminergic neurons are likely to be the first to undergo cell death due to their pre-existing state of oxidative stress. The death of other types of cells may have contributed to the continued high level of LDH in the combined 6-OHDA and SP group at the later days post-treatment. Alternatively, the combined treatment may have initiated a metabolic cascade that was not present in the other groups, thereby leading to delayed cell death.

When the NK$_1$ receptor antagonist NAT was combined with 6-OHDA, a small rise in SP expression occurred at 1 hour, which was comparable to the elevation produced by 6-OHDA alone. NAT treatment would not have been expected to prevent this initial rise generated by 6-OHDA since it does not affect the initial release of SP. The antagonist inhibits the downstream detrimental effects of SP that are a consequence of the ligand binding to its NK$_1$ receptor. Despite this, by day 1 post-treatment, SP content in this group was markedly less than that observed in the 6-OHDA alone and control groups. The slight but non-significant elevation of SP may have been due to antagonism of the NK$_1$ autoreceptor, known to be involved in the inhibition of SP release (Harrison and Geppetti, 2001). There was also an associated reduction in LDH concentration in the cell culture. Thus by blocking the effects of SP, cell death was reduced, further supporting a role for SP in cell death in vitro.

3.5 Conclusions

In conclusion, treatment with 6-OHDA in nigro-striatal organotypic culture increased SP
production and exacerbated cell death, as demonstrated by increased LDH content. This SP and LDH production was further increased when SP was combined with the 6-OHDA treatment, yet was reduced when 6-OHDA was combined with NAT treatment. These results indicate that elevated or higher than basal levels of SP may promote cell death \textit{in vitro}, a mechanism that can be blocked using an NK\textsubscript{1} receptor antagonist. Accordingly, the role of SP in dopaminergic cell death will be further investigated in the intrastriatal 6-OHDA experimental rodent model of PD following characterization of this PD model in the subsequent chapter.